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| <b>(21) International Application Number:</b> PCT/US99/06860<br><b>(22) International Filing Date:</b> 29 March 1999 (29.03.99)<br><br><b>(30) Priority Data:</b><br>09/049,719 27 March 1998 (27.03.98) US<br><br><b>(71) Applicants:</b> THE BOARD OF TRUSTEES OF THE LELAND STANFORD JR. UNIVERSITY [US/US]; 900 Welch Road, Palo Alto, CA 94304 (US). SRI INTERNATIONAL [US/US]; 333 Ravenswood Avenue, Menlo Park, CA 94025 (US).<br><br><b>(72) Inventors:</b> DENKO, Nicholas, C.; 573 9th Avenue, Menlo Park, CA 94025 (US). GIACCIA, Amato, J.; 1 Ryan Court, Stanford, CA 94305 (US). GREEN, Christopher, J.; 2737 Topaz Drive, Novato, CA 94945 (US). LADERROUTE, Keith, R.; 461 Forest Avenue, Palo Alto, CA 94301 (US). SCHINDLER, Cornelia; 922 Matadero Avenue, Palo Alto, CA 94306 (US). KOONG, Albert, Ching-Wei; 62 South Clark Avenue, Los Altos, CA 94024 (US).<br><br><b>(74) Agents:</b> CHOW, Y., Ping et al.; Heller Ehrman White & McAuliffe, 525 University Avenue, Palo Alto, CA 94301 (US).  | <b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).<br><br><b>Published</b><br><i>With international search report.</i><br><br><b>(88) Date of publication of the international search report:</b> 20 January 2000 (20.01.00) |   |
| <b>(54) Title:</b> HYPOXIA-INDUCIBLE HUMAN GENES, PROTEINS, AND USES THEREOF<br><br><b>(57) Abstract</b><br><br>The polynucleotide and polypeptide sequences of two novel hypoxia-inducible human genes, <i>HIG1</i> and <i>HIG2</i> , are described. In addition, a number of known genes have now been established as being hypoxia-inducible. These genes include <i>annexin V</i> , <i>lipocortin 2</i> , <i>hnRNP A1</i> , <i>Ku autoantigen</i> , <i>phosphoribosylpyrophosphate synthetase</i> , <i>acetoacetylCoA thiolase</i> , <i>ribosomal L7</i> , <i>fibroblast growth factor-3</i> , <i>EPH receptor ligand</i> , <i>plasminogen activator inhibitor-1</i> , <i>macrophage migration inhibitory factor</i> , <i>fibronectin receptor</i> , <i>lysyl hydroxylase-2</i> , <i>endothelin-2</i> , <i>B-cell translocation gene-1</i> , <i>reducing agent and tunicamycin-responsive protein</i> , <i>CDC-like kinase-1</i> , <i>quiescin</i> , <i>growth arrest DNA damage-inducible protein 45</i> , <i>DEC1</i> , <i>low density lipoprotein receptor related protein</i> , <i>hamster hairy gene homologue</i> , <i>adipophilin</i> , <i>cyclooxygenase-1</i> , <i>fructose bisphosphatase</i> , <i>creatine transporter</i> , <i>fatty acid binding protein</i> , <i>lactate dehydrogenase</i> , <i>Bcl-2-interacting killer</i> , <i>Nip3L/Nix</i> , and <i>Pim-1</i> . Polynucleotide and polypeptide arrays comprising the hypoxia-inducible gene sequences, proteins, or antibodies which specifically bind the proteins are disclosed. Methods for using the hypoxia-inducible gene sequences and proteins, and arrays thereof, to diagnose and treat hypoxia-related conditions such as cancer and ischemia are also provided. |  |   |

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## HYPOXIA-INDUCIBLE HUMAN GENES, PROTEINS, AND USES THEREOF

## BACKGROUND OF THE INVENTION

## 5 a) Field of the Invention

The present invention relates to hypoxia-inducible genes, and fragments thereof, and to the use of these sequences in the diagnosis and treatment of disease conditions involving hypoxia, including stroke, heart attack, and cancer.

## 10 b) Description of Related Art

Hypoxia is responsible for regulating a number of cellular and systemic processes, including angiogenesis, erythropoiesis, and glycolysis. Hypoxic insult and hypoxia-induced gene expression also play a role in a variety of severe pathological conditions including ischemia, retinopathy, neonatal distress, and  
15 cancer.

Hypoxia-induced gene expression is associated with ischemia (and reperfusion) in many tissues including the liver, heart, eyes, and brain. Many of the hypoxia-induced genes are believed to be involved in the protection or repair of the cells exposed to hypoxia. Enhancement of the body's protective expression  
20 of some stress-induced genes is therefore likely to be beneficial in many ischemia/reperfusion-related conditions such as liver transplantation, bypass operations, cardiac arrest, and stroke. For instance, in the brain, the response to brain ischemia includes the enhanced expression of growth factors and anti-apoptosis genes (Koistinaho et al. (1997) *Neuroreport* 20:i-viii).

25 However, the ischemic induction of gene expression is not always favorable. For example, brain ischemia can also result in the expression of apoptosis genes or other genes which promote degeneration of the neuronal cells. Ischemia can also induce an extreme inflammatory reaction in the injured brain via the upregulation of proinflammatory cytokines, chemokines, and endothelial-

leukocyte adhesion molecules (Feuerstein et al (1997) *Ann. N.Y. Acad.Sci.* 15:179-93). There is some evidence that this hypoxia-induced inflammatory response is a major cause of brain damage.

Eye diseases associated with neovascularization also involve hypoxia.

5 These eye diseases include diabetic retinopathy, retinopathy of prematurity, and sickle cell retinopathy. All can be serious enough to lead to blindness. The feasibility of treatment of retinopathy of prematurity by antisense inhibition of a hypoxia-induced gene, vascular endothelial growth factor (VEGF), has been demonstrated (Robinson, Patent No. 5,661,135).

10 The process of wound healing also involves the induction of gene expression by hypoxia (Anderson et al., Patent No. 5,681,706). TNF- $\alpha$  (tumor necrosis factor- $\alpha$ ) expression and secretion by macrophages is one response involved in wound healing that is induced by low oxygen. Other hypoxia-induced effects include the formation of scar tissue.

15 In addition to playing a major regulatory role in the body's response to stress in postnatal life, tissue hypoxia is responsible for regulating expression of genes in the developing embryo, particularly with regard to angiogenesis and vasoformation (Iyer et al. (1998) *Genes and Development* 12:149-162; Maltepe et al. (1997) *Nature* 386:403-407). Hypoxia also plays a role in neonatal stress and pregnancy-related diseases. For instance, oxygen tension appears to regulate  
20 cytotrophoblast proliferation and differentiation within the uterus (Genbacev et al. (1997) *Science* 277:1669-1672). Some disease conditions related to pregnancy, such as preeclampsia, are associated with abnormal cytotrophoblast differentiation and behavior. A number of studies have shown that an increased concentration of  
25 a hypoxia-induced gene product, insulin-like Growth Binding Protein (IGFBP-1), is associated with preeclampsia once manifest in the third trimester, even though US Patent No. 5,712,103 teaches that reduced levels of IGFBP-1 in maternal



blood in the first and second trimester, especially during the middle of the second trimester, can be used as a predictive indicator of preeclampsia.

Hypoxia has also been established to play a key role in neoplastic tissues. The progression of human tumors to malignancy is an evolutionary process  
5 involving the differential expression of multiple genes in response to unique microenvironments. Low oxygen conditions create a dominant tumor microenvironment which directly favors processes driving malignant progression, such as angiogenesis or elimination of p53 tumor suppressor activity.

In addition to promoting further tumor growth, the abnormally low oxygen  
10 levels that are found in nearly all solid tumors negatively impact therapeutic efforts. Hypoxic tumors often demonstrate resistance to radiation therapy and chemotherapy.

The connection between tumor hypoxia and the treatment of cancer is further exemplified by a study of cervical cancer that showed that the oxygen level  
15 of a tumor was an independent prognostic factor (Hoeckel et al. (1996) *Semin. Radiat. Oncol.* 6:1-8). The prognostic value of the oxygen level of a tumor was found to be more significant than all other indicators such as the age of the patient, clinical stage, or tumor size.

A number of oxygen-regulated genes have been identified in the art.  
20 Expression of many of these genes is induced by the interaction of hypoxia inducible factor-1 (HIF-1), a transcription factor complex, with the factor's DNA recognition site on the gene, the hypoxia-responsive element (HRE). HIF-1 has been cloned and found to not be activated by stressors such as heat shock and ionizing radiation.

25 Differential-display polymerase chain reaction (PCR) has been used to identify additional genes induced by hypoxia (O'Rourke et al. (1996) *Eur. J. Biochem.* 241:403-410). Six hypoxia-induced genes were identified, three of which were of known function. In addition to the known genes, two expressed

sequence tags (ESTs), and one full-length sequence were identified. The differential-display PCR method used by O'Rourke et al. to screen for hypoxically induced genes was found to be limited in its ability to identify hypoxically-induced genes.

5 In addition to the identification of hypoxia-induced genes, the identification of the stress-responsive regulatory elements of those genes is also of interest. The identification of such regulatory elements may provide for an inherently tumor-specific form of gene therapy. The HRE from a previously identified hypoxically induced gene, mouse phosphoglycerate kinase-1, has been used to control  
10 expression of heterologous genes both *in vitro* and *in vivo* (within a tumor) under hypoxic conditions (Dachs et al. (1997) Nature Medicine 3: 515-520). Similarly, a method for utilizing an anoxia-responsive element to effect controlled expression of a heterologous protein has been reported (Anderson et al., Patent No. 5,681,706).

15

## SUMMARY OF THE INVENTION

The present invention relates to genes whose expression is induced under hypoxic conditions.

One aspect of the present invention provides the isolated polynucleotide  
20 having the sequence shown as SEQ ID NO:1 (Fig. 1A), comprising the cDNA of the hypoxia-induced human gene *HIG1*, and encoding the polypeptide sequence of SEQ ID NO:2 (*HIG1*; Fig. 1B). Polynucleotides with sequences complementary to SEQ ID NO:1, fragments of SEQ ID NO:1 which are at least twelve nucleotides in length, and sequences which hybridize to SEQ ID NO:1 are also contemplated  
25 by the present invention. In particular, one aspect of the invention concerns the fragment of the sequence set forth in SEQ ID NO:1 comprising nucleotides 62-343, the nucleotides representing the coding sequence of human *HIG1*. The complements to the coding sequence, at least twelve nucleotide-long fragments of

the coding sequence, and sequences which hybridize to the coding sequence of *HIG1* are also provided by the invention.

Another aspect of the present invention provides the isolated polynucleotide having the sequence shown as SEQ ID NO:3 (Fig. 2A), comprising  
5 the cDNA of the hypoxia induced gene *HIG2*, and encoding the polypeptide sequence of SEQ ID NO:4 (*HIG2*; Fig. 2B). The complements to SEQ ID NO:3, as well as at least twelve nucleotide-long fragments thereof and sequences which hybridize thereto are also provided. The invention refers in particular to a polynucleotide having a sequence corresponding to nucleotides 274-465 of the  
10 sequence set forth in SEQ ID NO:3, or complements thereof, or at least twelve nucleotide-long fragments thereof, or sequences which hybridize thereto. Nucleotides 274-465 represent the coding sequence of human *HIG2*.

The present invention also encompasses expression vectors and delivery vehicles which contain polynucleotides of the present invention and host cells that  
15 are genetically engineered with polynucleotides of the present invention.

In another embodiment, the invention provides for an oligonucleotide probe comprising fragments, preferably at least about 15 nucleotides long, of the polynucleotides of SEQ ID NO:1 or SEQ ID NO:3, or the complement thereto.

Polypeptides of the sequences set forth in SEQ ID NO:2 (*HIG1*) and SEQ  
20 ID NO:4 (*HIG2*), or biochemically equivalent fragments of the polypeptides of either sequence, are further contemplated by the present invention.

Antibodies that are specifically immunoreactive to the hypoxia-induced polypeptides *HIG1* or *HIG2* of the present invention are also provided.

In still another embodiment, the present invention provides for arrays of  
25 polynucleotides or polypeptides corresponding to at least two different hypoxia-inducible genes, hypoxia-induced polypeptides, or antibodies immunoreactive with hypoxia-induced polypeptides.

Hypoxia-inducible genes suitable for use in the arrays, diagnostic methods, and treatment methods of the invention described herein are not limited to *HIG1* and *HIG2*, or derivatives thereof, but also include a number of known genes now determined to be hypoxia-inducible. Additional hypoxia-induced genes useful in the methods and arrays of the present invention include, but are not limited to, the genes of *annexin V*, *lipocortin 2*, *hnRNP A1*, *Ku autoantigen*, *phosphoribosylpyrophosphate synthetase*, *acetoacetylCoA thiolase*, *ribosomal L7*, *fibroblast growth factor-3*, *EPH receptor ligand*, *plasminogen activator inhibitor-1*, *macrophage migration inhibitory factor*, *fibronectin receptor*, *lysyl hydroxylase-2*, *endothelin-2*, *B-cell translocation gene-1*, *reducing agent and tunicamycin-responsive protein*, *CDC-like kinase-1*, *quiescin*, *growth arrest DNA damage-inducible protein 45*, *DEC1*, *low density lipoprotein receptor related protein*, *hamster hairy gene homologue*, *adipophilin*, *cyclooxygenase-1*, *fructose biphosphatase*, *creatine transporter*, *fatty acid binding protein*, *lactate dehydrogenase*, *Bcl-2-interacting killer*, *Nip3L/Nix*, and *Pim-1*.

In one aspect, the present invention provides diagnostic and prognostic tools for assaying for the expression of hypoxia-inducible genes in a tissue of an animal, for determining the presence of hypoxia in a tissue in an animal, and for evaluating a hypoxia-related condition in an animal particularly in order to tailor therapy to a known hypoxic state. The detection of expression products, such as mRNA transcripts or proteins, of the hypoxia-inducible genes of *HIG1*, *HIG2*, *annexin V*, *lipocortin 2*, *hnRNP A1*, *Ku autoantigen*, *phosphoribosylpyrophosphate synthetase*, *acetoacetylCoA thiolase*, *ribosomal L7*, *fibroblast growth factor-3*, *EPH receptor ligand*, *plasminogen activator inhibitor-1*, *macrophage migration inhibitory factor*, *fibronectin receptor*, *lysyl hydroxylase-2*, *endothelin-2*, *B-cell translocation gene-1*, *reducing agent and tunicamycin-responsive protein*, *CDC-like kinase-1*, *quiescin*, *growth arrest DNA damage-inducible protein 45*, *DEC1*, *low density lipoprotein receptor related*

*protein, hamster hairy gene homologue, adipophilin, cyclooxygenase-1, fructose biphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, or Pim-1, or combinations*

thereof, to determine the presence of hypoxia in a tissue or evaluate a hypoxia-

5 related condition in an animal is encompassed by the present invention. Methods of diagnosing and treating hypoxia-related conditions via such methods are also encompassed by the present invention.

Other methods of assaying for expression of hypoxia-inducible genes, determining the presence of hypoxia in a tissue in an animal, or evaluating a  
10 hypoxia-related condition in an animal involves the use of the arrays of the invention. First, a polynucleotide array or antibody array of the invention may be contacted with polynucleotides or polypeptides, respectively, either from or derived from a sample of body fluid or tissue obtained from the animal. Next, the amount and position of polynucleotide or polypeptide from the animal's sample  
15 which binds to the sites of the array is determined. Optionally, the gene expression pattern observed may be correlated with an appropriate treatment.

Other aspects of the invention concern treating a tissue which is a tumor by first determining the presence of hypoxia in the tumor and, second, treating the tumor with an established form of therapy for cancers such as radiation therapy,  
20 chemotherapy, and surgery.

In other aspects, the invention provides for methods of attenuating the hypoxic response of a tissue by blocking expression of a hypoxia-inducible gene *HIG1, HIG2, annexin V, lipocortin 2, hnRNP A1, Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7,*  
25 *fibroblast growth factor-3, EPH receptor ligand, plasminogen activator inhibitor-1, macrophage migration inhibitory factor, fibronectin receptor, lysyl hydroxylase-2, endothelin-2, B-cell translocation gene-1, reducing agent and tunicamycin-responsive protein, CDC-like kinase-1, quiescin, growth arrest DNA*

*damage-inducible protein 45, DEC1, low density lipoprotein receptor related protein, hamster hairy gene homologue, adipophilin, cyclooxygenase-1, fructose biphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, or Pim-1* in the cell or by

5 neutralizing the polypeptide expression products of these genes in the tissue. The invention also provides for methods of treating hypoxia-related conditions by attenuating the hypoxic response of a tissue in an animal such as a human.

Methods for enhancing the response of tissue to hypoxia are provided in other embodiments of the present invention. These methods involve  
10 administering expression vectors comprising the hypoxia-inducible genes of the present invention or administering polypeptide expression products of hypoxia-inducible genes to the tissue.

Methods for identifying stress-inducible and stress repressible genes are also provided.

15

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the human HIG1 cDNA and protein sequences. The nucleotide sequence for the human *HIG1* gene is shown in Figure 1A from 5' to 3' (SEQ ID NO:1). The coding sequence is underlined. The other regions are untranslated  
20 regions (5' and 3' UTR) of the gene. The protein sequence of human HIG1 is shown in Figure 1B (SEQ ID NO:2).

Figure 2 shows the human HIG2 cDNA and protein sequences. The nucleotide sequence for the human *HIG2* gene is shown in Figure 2A from 5' to 3' (SEQ ID NO:3). The coding sequence is underlined. The other regions are untranslated  
25 regions (5' and 3' UTR) of the gene. The protein sequence of human HIG2 is shown in Figure 2B (SEQ ID NO:4).

Figure 3 shows the murine HIG1 cDNA and protein sequences. The nucleotide sequence for the murine *HIG1* gene is shown in Figure 3A from 5' to 3' (SEQ ID NO:5). The coding sequence is underlined. The other regions are untranslated regions (5' and 3' UTR) of the gene. The protein sequence of murine HIG1 is shown in Figure 3B (SEQ ID NO:6).

Figure 4 shows the HIG1 cDNA and protein sequences of *seriola quinqueradiata*. The nucleotide sequence for this fish *HIG1* is shown in Figure 4A from 5' to 3' (SEQ ID NO:7). The coding sequence is underlined. The other regions are untranslated regions (5' and 3' UTR) of the gene. The protein sequence of fish HIG1 is shown in Figure 4B (SEQ ID NO:8).

Figure 5 shows the murine HIG2 cDNA and protein sequences. The nucleotide sequence for the murine *HIG2* gene is shown in Figure 5A from 5' to 3' (SEQ ID NO:9). The coding sequence is underlined. The other regions are untranslated regions of the gene (5' and 3' UTR). The protein sequence of murine HIG2 is shown in Figure 5B (SEQ ID NO:10).

Figure 6 shows the alignment of human HIG1 and HIG2 protein sequences with the HIG1 and HIG2 sequences of other species. The HIG1 homologues from humans (hHIG1), mice (mHIG1), and fish (*seriola quinqueradiata*) (fHIG1 or GHL1) are aligned in Figure 6A; the HIG2 homologues from humans (hHIG2) and mice (mHIG2) are aligned in figure 6B.

25

Figure 7 schematically illustrates the addition of linkers to cDNA library fragments. The linker addition is followed by PCR amplification.

Figure 8 illustrates how the subtraction protocol is used to enrich the tester cDNA library with sequences unique to the tester cDNAs.

5

## DETAILED DESCRIPTION OF THE INVENTION

### a) Definitions and General Parameters

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

10 By the term "hypoxia" (or "hypoxic") is meant, for the purposes of the specification and claims, an environment of reduced oxygen tension such that the oxygen content is less than or equal to about 5%. In most cases, hypoxic tissue will have an oxygen content that is less than or equal to about 2%.

15 "Normoxic" or "oxic" conditions are conditions comprising a normal level of oxygen for that particular environment. Normoxic or oxic tissue typically has an oxygen content above about 5%.

The terms "hypoxia-induced" or "hypoxia-inducible" when referring to a gene means that the gene is expressed at a higher level when the host cell is exposed to hypoxic conditions than when exposed to normoxic conditions.

20 Typically, the number of mRNA transcripts of a hypoxia-induced gene would be at least about 20% higher in a hypoxic cell versus a normoxic cell. Preferably, expression of the hypoxia-induced gene is at least about 2-fold higher in hypoxic versus normoxic cells. Most preferably, expression of the hypoxia-inducible gene is at least about 5-fold higher in hypoxic cells versus normoxic cells.

25 A "hypoxia-related condition" in an animal is a condition where hypoxia or altered (typically, enhanced) levels of expression of hypoxia-inducible genes in a tissue of the animal is involved. The hypoxia or altered expression of hypoxia-inducible genes may either be a symptom or play a role in the cause, development,



progression, amelioration, or cure of the condition. A hypoxia-related condition may optionally be a disease or pathological condition. Hypoxia-related conditions include, but are not limited to, cancer, ischemia, reperfusion, retinopathy, neonatal distress, preeclampsia, cardiac arrest, stroke, and wound  
5 healing.

The term "hypoxia-induced protein" or "hypoxia-induced gene product" means a protein encoded by a gene whose expression is induced by hypoxia.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For  
10 example, naturally-occurring polynucleotides or polypeptides present in a living animal are not isolated, but the same polynucleotides or polypeptides could be part of a vector or composition, and be isolated in that such vector or composition is not part of its natural environment.

A "sample obtained from a patient" or a "sample obtained from an animal"  
15 may be a sample of tissue or a sample of body fluid. The term "tissue" is used herein to refer to any biological matter made up of one cell, multiple cells, an agglomeration of cells, or an entire organ. The term tissue, as used herein, encompasses a cell or cells which can be either normal or abnormal (i.e. a tumor). A "body fluid" may be any liquid substance extracted, excreted, or secreted from  
20 an organism or a tissue of an organism. The body fluid need not necessarily contain cells. Body fluids of relevance to the present invention include, but are not limited to, whole blood, serum, plasma, urine, cerebral spinal fluid, tears, and amniotic fluid.

The term "biochemically equivalent variations" means protein or nucleic  
25 acid sequences which differ in some respect from the specific sequences disclosed herein, but nonetheless exhibit the same, or substantially the same, functionality. In the case of cDNA, for example, this means that modified sequences which contain other nucleic acids than those specifically disclosed are encompassed,

provided that the alternate cDNA encodes mRNA which in turn encodes a protein of this invention. Such modifications may involve the substitution of only a few bases, or many. The modifications may involve substitution of degenerate coding sequences or replacement of one coding sequence with another; introduction of  
5 non-natural nucleic acids is contemplated. It is not necessary for the alternate DNA to hybridize with that disclosed herein provided that the functional criterion is met. Preferably, the modified nucleic acid sequence hybridizes to and is at least 95% complementary to the sequence of interest.

Similarly, in the case of the proteins of this invention, alterations in the  
10 amino acid sequence which do not affect functionality may be made. Such variations may involve replacement of one amino acid with another, use of side chain modified or non-natural amino acids, and truncation. The skilled artisan will recognize which sites are most amenable to alteration without affecting the basic function.

15 A "polynucleotide", "oligonucleotide", or "nucleic acid" includes, but is not limited to, mRNA, cDNA, genomic DNA, and synthetic DNA and RNA sequences, comprising the natural nucleoside bases adenine, guanine, cytosine, thymine, and uracil. The term also encompasses sequences having one or more modified nucleosides. The terms "polynucleotide" and "oligonucleotide" are used  
20 interchangeably herein. No limitation as to length or to synthetic origin are suggested by the use of either of these terms herein.

The term "polypeptide" means a poly(amino acid) comprising at least two amino acids linked by peptide bonds. A "protein" is a polypeptide which is encoded by a gene.

25 "Neutralizing" a polypeptide or protein means inhibiting, partially or wholly, the bioactivity of the polypeptide or protein. This inhibition of activity may mean inhibition of catalytic activity, prevention of binding to a receptor or ligand, blockage or dimer formation, or the like.

The term "sequences which hybridize thereto" means polynucleotide sequences which are capable of forming Watson-Crick hydrogen bonds with another polynucleotide sequence under normal hybridization conditions, such as in buffered (pH. 7.0-7.5) aqueous, saline solutions (for instance, 1 to 500 mM NaCl) at room temperature. Although normal hybridization conditions will depend on the length of the polynucleotides involved, typically they include the presence of at least one cation such as  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ , or  $\text{Ca}^{2+}$ , a near neutral pH, and temperatures less than 55°C. Although the sequences which hybridize to a polynucleotide may be about 90%-100% complementary to the polynucleotide, if the sequences are of sufficient length, in solutions with high salt concentrations, and/or under low temperature conditions, polynucleotides with complementarity of 70% or above, or even just 50% or above, may hybridize to the polynucleotide. Sequences which hybridize thereto typically comprise at least 12 nucleotides, and preferably at least about 15 nucleotides, which are complementary to the target polynucleotide.

A "coding sequence" is a polynucleotide or nucleic acid sequence which is transcribed and translated (in the case of DNA) or translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A transcription termination sequence will usually be located 3' to the coding sequence.

Nucleic acid "control sequences" refer to translational start and stop codons, promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, as necessary and sufficient for the transcription and translation of a given coding sequence in a defined host cell. Examples of control sequences suitable for eucaryotic cells are promoters, polyadenylation signals, and enhancers. All of

these control sequences need not be present in a recombinant vector so long as those necessary and sufficient for the transcription and translation of the desired gene are present.

“Operably or operatively linked” refers to the configuration of the coding  
5 and control sequences so as to perform the desired function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. A coding sequence is operably linked to or under the control of transcriptional regulatory regions in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence  
10 into mRNA that can be translated into the encoded protein. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered “operably linked” to  
15 the coding sequence.

The expression products described herein may consist of proteinaceous material having a defined chemical structure. However, the precise structure depends on a number of factors, particularly chemical modifications common to proteins. For example, since all proteins contain ionizable amino and carboxyl  
20 groups, the protein may be obtained in acidic or basic salt form, or in neutral form. The primary amino acid sequence may be derivatized using sugar molecules (glycosylation) or by other chemical derivatizations involving covalent or ionic attachment with, for example, lipids, phosphate, acetyl groups and the like, often occurring through association with saccharides. These modifications may occur *in vitro*, or *in vivo*, the latter being performed by a host cell through posttranslational  
25 processing systems. Such modifications may increase or decrease the biological activity of the molecule, and such chemically modified molecules are also intended to come within the scope of the invention.

“Vector” means a polynucleotide comprised of single strand, double strand, or circular DNA or RNA. An “expression vector” is comprised of the following elements operatively linked at appropriate distances for allowing functional gene expression: replication origin, promoter, enhancer, 5’ mRNA leader sequence, ribosomal binding site, nucleic acid cassette, termination and polyadenylation sites, and selectable marker sequences. One or more of these elements may be omitted in specific applications. The nucleic acid cassette can include a restriction site for insertion of the nucleic acid sequence to be expressed. In a functional vector the nucleic acid cassette contains the nucleic acid sequence to be expressed including translation initiation and termination sites. An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the “control” of the control sequences. Modification of the sequences encoding the particular protein of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; or to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site which is in reading frame with and under regulatory control of the control sequences.

A “regulatory element” is a segment of DNA to which a transcription factor(s) binds and alters the activity of a gene’s promoter either positively (induction) or negatively (repression).

A “stress-responsive element” or “stress-responsive regulatory element” is a regulatory element which binds transcription factors activated by the cell in

response to environmental stress. Environmental stressors may include one or more of the following: oxygen depletion; radiation; heat shock; pH change; hypothermia; or glucose starvation.

A "delivery vehicle", as used herein, refers to a means of delivering a polypeptide or a polynucleotide to a cell. The delivery vehicle is preferably used to deliver an expression vector to a cell or a cell in an organism. A delivery vehicle may be a virus, such as a retrovirus, an adenovirus, an adeno-associated virus, a herpes simplex virus, or a vaccinia virus.

Other possible delivery vehicles are non-viral. For instance, one of the many liposome formulations known to those skilled in the art, such as Lipofectin, may serve as a delivery vehicle. Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have internal aqueous space useful for entrapping water soluble compounds such as polynucleotides. Recognition molecules can be attached to their surface for the targeting of the delivery vehicles to specific tissues.

As used herein, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. Antibodies may exist as intact immunoglobulins or as a number of fragments, including those well-characterized fragments produced by digestion with various peptidases. While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that antibody fragments may be synthesized de novo either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized de novo using recombinant DNA methodologies. Antibody fragments encompassed by the use of the term "antibodies" include, but are not limited to, Fab, Fab', F(ab')<sub>2</sub>, scFv, Fv, dsFv diabody, and Fd fragments.

The phrase “specifically binds to a polypeptide” or “specifically immunoreactive with”, when referring to an antibody refers to a binding reaction which is determinative of the presence of the polypeptide (or protein) in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions may require an antibody that is selected for its specificity for a particular protein or polypeptide. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein.

b) Hypoxia-Inducible Genes and Expression Products

We have discovered a novel human gene, herein referred to as *HIG1*, whose expression is induced by cellular response to hypoxia (see the specific examples, Examples 1-6 below). We have isolated a cDNA of the human *HIG1* gene (SEQ ID NO:1; Fig. 1A) and identified the coding sequence to be nucleotides 62-343 of SEQ ID NO:1. The protein encoded by *HIG1* comprises the amino acid sequence shown in Figure 1B (SEQ ID NO:2). Polynucleotides with sequences complementary to SEQ ID NO:1, polynucleotides that are fragments of SEQ ID NO:1 of at least twelve nucleotides in length and polynucleotides which hybridize to SEQ ID NO:1 are also within the scope of the present invention. The fragments of SEQ ID NO:1 are preferably at least 15 nucleotides long.

In particular, polynucleotides comprising the nucleotides 62-343 of SEQ ID NO:1, or complements thereto, or at least twelve nucleotide long fragments thereof, or sequences which hybridize thereto are preferred. Fragments of the coding sequence of *HIG1* are preferably at least fifteen nucleotides in length.

We have also discovered a second, novel human gene, herein referred to as *HIG2*, whose expression is induced by cellular response to hypoxia. We have isolated a cDNA clone of this gene. The cDNA sequence of the *HIG2* gene is shown in Fig. 2A (SEQ ID NO:3). The coding sequence of *HIG2* comprises  
5 nucleotides 274-465 of SEQ ID NO:3. Fragments of the *HIG2* sequence, and of the *HIG2* coding sequence in particular, of at least twelve, and preferably fifteen, nucleotides in length are provided by the present invention as well.

Polynucleotides of sequence which is complementary to SEQ ID NO:3 (especially to nucleotides 274-465) or polynucleotides which hybridize to polynucleotides of  
10 the sequence set forth in SEQ ID NO:3 (especially to nucleotides 274-465), are also contemplated.

Polypeptides encoded by the polynucleotides of *HIG1* (SEQ ID NO:2; Fig. 1B) and *HIG2* (SEQ ID NO:4; Fig. 2B), or biochemically equivalent variations of either protein, are also provided by the present invention. Fragments of these  
15 polypeptides which consist of at least eight amino acids are provided as well. Preferably, the fragments are at least 15 amino acids in length.

All biochemically equivalent variations of the aforementioned polynucleotides and polypeptides are considered to be fully within the scope of this invention. The mouse and fish *HIG1* polynucleotide and polypeptide  
20 sequences (Figs. 3, 4, and 6) can be considered biochemically equivalent variations of the human *HIG1*. The mouse *HIG2* polynucleotide and polypeptide sequences (Figs. 5 and 6) are likewise understood to be biochemically equivalent variations of the human *HIG1*.

The polynucleotides of this invention may readily be incorporated within  
25 expression vectors by one of ordinary skill in the art. In a preferred embodiment, the polynucleotide sequence comprising nucleotides 62-343 of SEQ ID NO:1 (the coding sequence of *HIG1*) or nucleotides 274-465 of SEQ ID NO:2 (the coding



sequence of HIG2) is operably linked with appropriate control sequences, such as a promoter.

Alternatively, larger fragments of the polynucleotides of SEQ ID NO:1 or SEQ ID NO:2 which comprise portions of the untranslated regions of the genes  
5 may be used in an expression vector instead. This may be particularly useful when hypoxia-inducibility is desired, since the untranslated regions may contain critical regulatory regions such as hypoxia-responsive elements.

The polynucleotides of this invention may also be incorporated within a host cell. In one embodiment, transfection may be used to introduce an expression  
10 vector containing one of the polynucleotides of the invention into the cell. The polynucleotide of the transfected vector may also be operably linked with control sequences including regulatory elements to effect the expression within the cell of exogenous protein or polypeptide sequences encoded by the polynucleotides of the present invention. Methods of cloning, amplification, expression, and purification  
15 will be apparent to the skilled artisan. Representative methods are disclosed in *Molecular Cloning: a Laboratory Manual, 2nd Ed., Vol. 1-3*, eds. Sambrook et al., Cold Spring Harbor Laboratory (1989).

A *HIG1* or *HIG2* polynucleotide may be introduced into an animal either by first incorporating the vector into a cell and then transferring the cell to the  
20 animal (*ex vivo*) or by incorporating the vector into a cell within an animal directly (*in vivo*).

The introduction of a *HIG1* or *HIG2* polynucleotide into a cell may be achieved by directly injecting the nucleic acid into the cell or by first mixing the nucleic acid with polylysine or cationic lipids which will help facilitate passage  
25 across the cell membrane. However, introduction of the polynucleotide into the cell is preferably achieved through the use of a delivery vehicle such as a liposome or a virus. Viruses which may be used to introduce a *HIG1* or *HIG2* polynucleotide or expression vector into a cell include, but are not limited to,

retroviruses, adenoviruses, adeno-associated viruses, herpes simplex viruses, and vaccinia viruses.

Antisense oligonucleotides complementary to *HIG1* and *HIG2*, particularly those which are capable of blocking expression of *HIG1* or *HIG2* are provided by the present invention. The antisense oligonucleotide is preferably an oligonucleotide having a sequence complementary to at least a portion (preferably at least about 12 nucleotides in length) of SEQ ID NO:1 or SEQ ID NO:3. The antisense oligonucleotide is preferably between about 15 and about 22 nucleotides in length. Modifications of the sequence or bases of the antisense oligonucleotide may be desirable to facilitate transfer into a cell, stability, or tight binding to the *HIG1* or *HIG2* mRNA.

An oligonucleotide probe is provided by another embodiment of the invention. The probe consists of one of the polynucleotides of this invention, or an at least 12 nucleotide-long fragment thereof. The probe may be used to assay for, and if the probe is properly labeled, quantitate, the hypoxia-induced expression of *HIG1* or *HIG2* in a cell. In a preferred embodiment, the probe is at least about 15 nucleotides in length. In a particularly preferred embodiment, the probe is between 15 and 22 nucleotides in length.

Antibodies specifically immunoreactive with the *HIG1* or *HIG2* polypeptides represent still another embodiment of the invention. These antibodies may be monoclonal or polyclonal. The antibodies may optionally be recombinant or purely synthetic. The antibody may be an intact antibody or fragment. The preparation of antibodies specific to the *HIG1* and *HIG2* polypeptides would be routine for those skilled in the art.

In addition to the identification of the new genes *HIG1* and *HIG2* which were found to be hypoxia-inducible, we have also established for the first time that several previously known genes are hypoxia-inducible in humans (see the specific examples, Examples 2 and 9, below). These genes include *annexin V*, *lipocortin*

2, *hnRNP A1*, *Ku autoantigen*, *phosphoribosylpyrophosphate synthetase*,  
*acetoacetylCoA thiolase*, *ribosomal L7*, *fibroblast growth factor-3*, *EPH receptor*  
*ligand*, *plasminogen activator inhibitor-1*, *macrophage migration inhibitory*  
*factor*, *fibronectin receptor*, *lysyl hydroxylase-2*, *endothelin-2*, *B-cell*  
5 *translocation gene-1*, *reducing agent and tunicamycin-responsive protein*, *CDC-*  
*like kinase-1*, *quiescin*, *growth arrest DNA damage-inducible protein 45*, *DEC1*,  
*low density lipoprotein receptor related protein*, *hamster hairy gene homologue*,  
*adipophilin*, *cyclooxygenase-1*, *fructose biphosphatase*, *creatine transporter*,  
*fatty acid binding protein*, *lactate dehydrogenase*, *Bcl-2-interacting killer*,  
10 *Nip3L/Nix*, and *Pim-1*. Furthermore, expression of glyceraldehyde-3-phosphate  
dehydrogenase (GAPDH), expression previously known to be hypoxia-inducible  
only in endothelial cells (Graven et al. (1998) Am. J. Physiol., 274(2 Pt 1):C347-  
355), is now shown by our work to be greatly induced in transformed cells.  
Additionally, a multitude of EST sequences from the databases have now been  
15 identified as being hypoxia-inducible (Table 3, Example 8 and Table 5, Example  
9).

c) Polynucleotide, Polypeptide, and Antibody Arrays.

Another aspect of the invention involves the presentation of multiple (at  
20 least two, and preferably more than four) hypoxia-inducible gene sequences,  
polynucleotide probes complementary to the hypoxia-inducible gene sequences,  
hypoxia-induced polypeptides, or antibodies (immunoreactive with hypoxia-  
induced polypeptides) on an array. In particularly preferred arrays, more than  
about 10 different polynucleotides, polypeptides, or antibodies are presented on  
25 the array. In an alternative preferred embodiment, the number of different  
polynucleotides, proteins, or antibodies on the array is greater than about 25, or  
even greater than about 100.

One aspect of the invention provides an array of polynucleotides which comprises at least two different hypoxia-inducible genes, or complements thereto, or at least twelve nucleotide-long fragments thereof, or sequences which hybridize thereto. The hypoxia-inducible genes or their fragments may optionally be  
5 selected from HIG1, HIG2, any of the hypoxia-inducible genes listed in Table 1 (below), Table 3 (Example 8, below), and Table 5 (Example 9, below). However, it is understood that all of the hypoxia-inducible gene sequences on the array need not be derived only from those hypoxia-inducible listed herein. The polynucleotides on the array are typically single-stranded.

10 For instance, in one embodiment of the polynucleotide array, one of the multiple polynucleotides on the array is derived from either the HIG1 or HIG2 gene sequences. The polynucleotides of the array may comprise the entire sequence of one strand of the gene, or may comprise at least 12 nucleotide long fragments thereof, or sequences which hybridized thereto. In an alternative  
15 embodiment, one of the polynucleotides of the array comprises a polynucleotide corresponding to nucleotides 62-343 of SEQ ID NO:1 (*HIG1*) or nucleotides 274-465 of SEQ ID NO:2 (*HIG2*), or complements to one of the coding sequences, or at least twelve nucleotide-long fragments of one of the coding sequences, or sequences which hybridize to one of the coding sequences. In another  
20 embodiment of the polynucleotide array, at least one of the polynucleotide sequences of *HIG1*, *HIG2*, *annexin V*, *lipocortin 2*, *hnRNP A1*, *Ku autoantigen*, *phosphoribosylpyrophosphate synthetase*, *acetoacetylCoA thiolase*, *ribosomal L7*, *fibroblast growth factor-3*, *EPH receptor ligand*, *plasminogen activator inhibitor-1*, *macrophage migration inhibitory factor*, *fibronectin receptor*, *lysyl*  
25 *hydroxylase-2*, *endothelin-2*, *B-cell translocation gene-1*, *reducing agent and tunicamycin-responsive protein*, *CDC-like kinase-1*, *quiescin*, *growth arrest DNA damage-inducible protein 45*, *DEC1*, *low density lipoprotein receptor related protein*, *hamster hairy gene homologue*, *adipophilin*, *cyclooxygenase-1*, *fructose*

*bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1* is represented on the array in combination with a second, different polynucleotide sequence from a hypoxia-inducible gene. The second polynucleotide sequence may be selected  
5 from *HIG1*, *HIG2*, any of the hypoxia-inducible genes represented in Table 1, shown below, any of the expressed sequence tags of hypoxia-inducible genes shown in Table 3 (see Example 8), or any other hypoxia-inducible gene or expressed sequence tag from a hypoxia-inducible gene. It is understood that regardless of which genes are represented on the array, the gene sequences do not  
10 have to be represented in their entirety.

The polynucleotide sequences that are immobilized on the array are most preferably, single-stranded and complementary to the mRNA transcripts of the relevant hypoxia-inducible genes. The immobilized polynucleotides may be fragments or complementary sequences of the gene or EST sequence that contain  
15 at least twelve nucleotides and preferably at least fifteen nucleotides. Alternatively, longer gene fragments including EST fragments of at least 50 or at least 100 nucleotides may be used. In a preferred embodiment of the array, the array is made up of many different gene sequences.

In another embodiment of the polynucleotide array, only polynucleotides  
20 correlating to hypoxia-inducible genes expressing gene products of a similar function are included on the array. At least two, but preferentially more than two, different hypoxia-induced genes encoding proteins from a single functional category are represented on the array. Examples of seven functional categories of hypoxia-inducible proteins are as follows: (1) glycolytic enzymes/proteins; (2)  
25 angiogenesis/tissue remodeling proteins; (3) erythropoiesis/vascular regulatory proteins; (4) metabolic/homeostatic proteins; (5) apoptosis proteins; (6) DNA repair proteins; and (7) cell-cycle proteins. These categories are shown in Table 1, below, along with some representative members of each of the categories. It is

understood that the members of each of the seven functional categories of hypoxia-inducible proteins are not limited to the lists shown in Table 1. It is further understood, that the list of functional categories of hypoxia-inducible genes is not limited to the seven categories listed in Table 1. Again, a preferred embodiment of this array comprises polynucleotide sequences complementary to the mRNA transcripts of the relevant hypoxia inducible genes. A particularly preferred embodiment of an array displays multiple polynucleotide sequences, each of which is complementary to a different gene which encodes a protein involved in angiogenesis and/or tissue remodeling.

10

**Table 1. Seven Functional Categories of Hypoxia-Inducible Genes**

| <b>GLYCOLYTIC ENZYMES/PROTEINS</b>                     |
|--|
| lactate dehydrogenase (LDH)                            |
| phosphoglycerate kinase (PGK)                          |
| aldolase A   |
| L-phosphofructokinase (PFKL)                           |
| glucose transporter isoform 3 (Glut-3)                 |
| interleukin-2  |
| glyceraldehyde-3-phosphate dehydrogenase (GAPDH)       |
| adenylate kinase isoenzyme 3 (AK-3)                    |
| <b>ANGIOGENESIS/TISSUE REMODELING PROTEINS</b>         |
| vascular endothelial growth factor (VEGF)              |
| platelet-derived growth factor $\beta$ (PDGF $\beta$ ) |
| transforming growth factor $\beta$ (TGF $\beta$ )      |

tumor necrosis factor  $\alpha$  (TNF $\alpha$ )

interleukin-6 (IL-6)

interleukin-2 (IL-2)

tissue factor

fibroblast growth factor (FGF-3)

EPH receptor ligand

plasminogen activator inhibitor-1 (PAI-1)

macrophage migration inhibitory factor (MIF)

fibronectin receptor

lysyl hydroxylase-2

endothelin-2

#### **ERYTHROPOIESIS/VASCULAR REGULATORY PROTEINS**

erythropoietin (EPO)

tyrosine hydroxylase

heme oxygenase

alpha-fetoprotein (AFP)

endothelin

#### **METABOLIC/HOMEOSTATIC PROTEINS**

insulin-like growth factor binding protein-1 (IGFBP-1)

metallothionein

creatine kinase

inducible nitric oxide synthase (*i*-NOS-1)

epidermal growth factor receptor (EGFR)  
huntingtin-associated protein 1 (HAP-1)  
glucose-regulated protein 78 (GRP78)  
glucose-regulated protein 90 (GRP90)  
thioredoxin  
annexin V  
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)  
heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1)  
gamma-glutamyl cysteine synthetase heavy subunit  
phosphoribosylpyrophosphate synthetase (PRPP synthetase)  
acetoacetylCoA thiolase  
fructose biphosphatase  
creatine transporter  
fatty acid binding protein  
glucose transporter isoform 3 (Glut-3)  
adenylate kinase isoenzyme 3 (AK-3)  
lactate dehydrogenase (LDH)

**APOPTOSIS PROTEINS**

insulin-like growth factor binding protein-3 (IGFBP-3)  
c-myc  
c-jun  
Bcl-2-interacting killer (BIK)



|  |
|--|
| 19 kDa-interacting protein 3 long/Nip3-like protein X (NipP3L/Nix)<br>Pim-1  |
| <b>DNA-REPAIR PROTEINS</b>   |
| Ku (70)  |
| <b>CELL-CYCLE PROTEINS</b>   |
| B-cell translocation gene-1 (BTG-1)<br>reducing agent and tunicamycin responsive protein (RTP)<br>CDC-like kinase-1 (clk-1)<br>quiescin (Q6)<br>growth arrest DNA damage-inducible protein 45 (GADD45) |

In an alternative embodiment of the polynucleotide array, polynucleotides correlating to the gene sequences encoding proteins belonging to at least two different functional categories of hypoxia-inducible genes are displayed on a single array. Although at least two different polynucleotide sequences are  
5 required to form the array, in a preferred embodiment many more than two are used. Again, a preferred embodiment of this array comprises polynucleotide sequences complementary to the mRNA transcripts of the relevant hypoxia inducible genes of at least 12 nucleotides in length, and preferably fifteen.

The present invention also provides for polypeptide arrays analogous to the  
10 polynucleotide arrays discussed above, except that the polypeptide sequences of the hypoxia-inducible genes, or fragments thereof, are displayed in an array. The polypeptide array comprises the polypeptide expression products of at least two hypoxia-inducible genes, or biochemically equivalent fragments thereof. For instance, the polypeptide array may comprise the protein HIG1 or HIG2 and at least  
15 one other protein which is a hypoxia induced gene product. Alternatively, the

polypeptide array may instead comprise at least one protein selected from the group consisting of HIG1, HIG2, annexin V, lipocortin 2, hnRNP A1, Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3, EPH receptor ligand, plasminogen  
5 activator inhibitor-1, macrophage migration inhibitory factor, fibronectin receptor, lysyl hydroxylase-2, endothelin-2, B-cell translocation gene-1, reducing agent and tunicamycin-responsive protein, CDC-like kinase-1, quiescin, growth arrest DNA damage-inducible protein 45, DEC1, low density lipoprotein receptor related protein, hamster hairy gene homologue, adipophilin, cyclooxygenase-1, fructose  
10 bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1, or a biochemically equivalent fragment thereof; and at least one of a second polypeptide which is a second hypoxia-induced gene product, or a biochemically equivalent fragment thereof.

15 Another aspect of the invention concerns a polypeptide array comprising at least two different hypoxia-induced proteins, or biochemically equivalent fragments thereof, wherein each hypoxia-induced protein belongs to a different functional category. Alternatively, the polypeptide array comprises at least two different hypoxia-induced proteins or biochemically equivalent fragments thereof,  
20 wherein said hypoxia-induced proteins are all proteins belonging to a single functional category. Optionally, the functional category may be selected from the group consisting of glycolytic enzymes/proteins, metabolic/homeostatic proteins, apoptosis proteins, DNA repair proteins, angiogenesis/tissue remodeling proteins, cell-cycle proteins, and erythropoiesis/vascular regulatory proteins. (See Table 1,  
25 above).

Yet another alternative embodiment of the invention, is an array analogous to a polypeptide array described above, except that antibodies immunoreactive with the hypoxia-induced polypeptides are immobilized to form the array, rather

than the polypeptide sequences themselves. Each array comprises at least two different antibodies, each of which is immunoreactive with a different hypoxia-induced protein. Each of the two antibodies is specifically immunoreactive with the polypeptide expression products of hypoxia-inducible genes, such as, but not limited to, HIG1 or HIG2. For instance, in one embodiment, the antibody array comprises at least one antibody immunoreactive with a protein selected from the group consisting of HIG1, HIG2, annexin V, lipocortin 2, hnRNP A1, Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3, EPH receptor ligand, plasminogen activator inhibitor-1, macrophage migration inhibitory factor, fibronectin receptor, lysyl hydroxylase-2, endothelin-2, B-cell translocation gene-1, reducing agent and tunicamycin-responsive protein, CDC-like kinase-1, quiescin, growth arrest DNA damage-inducible protein 45, DEC1, low density lipoprotein receptor related protein, hamster hairy gene homologue, adipophilin, cyclooxygenase-1, fructose biphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1. The antibody array further comprises at least one of a second antibody, wherein said second antibody specifically binds a second hypoxia-induced gene product or a biochemically equivalent fragment thereof.

The antibodies on the array may be monoclonal or polyclonal. They may be intact antibodies or fragments of antibodies that are capable of specifically binding the polypeptides of the present invention. As is the case with the polynucleotide and polypeptide arrays of the invention, the antibody array preferably comprises at least four different antibodies, and preferably more than about 10 different antibodies.

Methods of constructing arrays of biomolecules, especially polynucleotides, have been previously established in the art. For instance, some methods for preparing particularly high density polynucleotide arrays are

disclosed in Pirrung et al., Patent No. 5,143,854, Pirrung et al., Patent No. 5,405,783, and Fodor et al., Patent No. 5,510,270, all of which are herein incorporated by reference. The polypeptides, antibodies, or polynucleotides may be immobilized on the array either covalently or noncovalently. Methods for  
5 immobilizing biomolecules are well known to those of ordinary skill in the art. The material to which the polynucleotides or polypeptides are immobilized in the array may vary. Possible substrates for construction of a biomolecule array include, but are not limited to, cellulose, glass, silicon, silicon oxide, silicon nitride, polystyrene, germanium, (poly)tetrafluorethylene, and gallium phosphide.

10

d) Methods of Use

In all of the methods of use described below, the animal is preferably a mammal. Most preferably, the mammal is a human.

We have demonstrated that the expression of *HIG1* or *HIG2* and a number  
15 of other genes is indicative of a cell's response to hypoxia as shown in the specific examples shown below (Examples 1-9). Accordingly, detection of abnormal levels of the transcripts of hypoxia-inducible genes such as *HIG1* or *HIG2*, or combinations thereof, in the tissues or body fluids of an animal can be used in both a diagnostic and prognostic manner for hypoxia-related conditions. The  
20 abnormal levels may be characterized by either increased levels or decreased levels, depending upon the hypoxia-related condition being analyzed. In other cases, either the complete absence or any presence of a hypoxia-inducible gene transcript may be indicative of an abnormal condition. Similarly, detection of abnormal levels of the hypoxia-induced polypeptides, or combinations thereof,  
25 can be used in either a diagnostic or prognostic manner for hypoxia-related conditions. The presence of hypoxia in a tissue can be evaluated by testing for the presence or absence of the transcripts or polypeptides encoded by the polynucleotides of the invention in either the tissue or in the body fluids of the

animal. Detection of the transcripts or polypeptides can be either qualitative or quantitative.

One aspect of the invention, therefore, provides a method of determining the presence of hypoxia in a tissue in an animal or evaluating a hypoxia-related condition in a tissue in an animal. These methods comprise assaying for either the messenger RNA (mRNA) transcripts or the polypeptide expression product of at least one gene selected from the group consisting of *HIG1*, *HIG2*, *annexin V*, *lipocortin 2*, *hnRNP A1*, *Ku autoantigen*, *phosphoribosylpyrophosphate synthetase*, *acetoacetylCoA thiolase*, *ribosomal L7*, *fibroblast growth factor-3*, *EPH receptor ligand*, *plasminogen activator inhibitor-1*, *macrophage migration inhibitory factor*, *fibronectin receptor*, *lysyl hydroxylase-2*, *endothelin-2*, *B-cell translocation gene-1*, *reducing agent and tunicamycin-responsive protein*, *CDC-like kinase-1*, *quiescin*, *growth arrest DNA damage-inducible protein 45*, *DEC1*, *low density lipoprotein receptor related protein*, *hamster hairy gene homologue*, *adipophilin*, *cyclooxygenase-1*, *fructose biphosphatase*, *creatine transporter*, *fatty acid binding protein*, *glucose transporter-like protein III*, *lactate dehydrogenase*, *Bcl-2-interacting killer*, *Nip3L/Nix*, and *Pim-1* in a body fluid or the tissue of the animal. This method determining the presence of hypoxia in a tissue may be used to diagnose a hypoxia-related condition in a animal.

The presence of hypoxia in a tissue or the degree of expression of hypoxia-inducible genes determined by these methods may be used to select an appropriate treatment for the animal. For instance, the hypoxia-related condition being evaluated may be cancer and the tissue which is the target of the evaluation may optionally be a tumor. The degree to which the tumor is showing gene expression patterns characteristic of hypoxia or the activation of genes involved in angiogenesis, for instance, can be usefully correlated with appropriate treatment of tumors of that particular type.

The hypoxia-related condition, however, need not necessarily be cancer. The hypoxia-related condition may instead be any condition in which hypoxic conditions play a role (favorable or detrimental to the animal). Such conditions include, but are not limited to, ischemia, reperfusion, retinopathy, neonatal  
5 distress, preeclampsia, cardiac arrest, stroke and wound healing.

The transcripts of hypoxia-inducible genes may be detected by any of several means known to those skilled in the art. One embodiment of diagnostic detection involves annealing to the transcript, *in vivo* or *in vitro*, a labeled nucleic acid probe complementary to the transcript sequence. The labeled probe can be  
10 fluorescent, radioactive, immunoreactive, colormetric or otherwise marked for detection. To detect very minute quantities of a transcript, amplification of the transcript in a tissue or fluid sample from the animal may first be performed to aid subsequent detection of the transcript. Amplification of the hypoxically-induced transcripts can be readily achieved using the polynucleotides of the present  
15 invention as primers, using reverse transcriptase to make a cDNA copy of the transcript, and then using polymerase chain reaction to achieve exponential amplification.

Detection of expression of the polypeptide products of the HIG1 or HIG2 genes, or any of the other hypoxia-induced genes could be achieved, for instance,  
20 by the application of labeled antibodies specifically immunoreactive with the polypeptide products. The antibodies can be applied to the tissue *in vivo*, or to tissue or body fluid samples removed from the animal. Various forms of typical immunoassays known to those skilled in the art would be applicable here. These assays include both competitive and non-competitive assays. For instance, in one  
25 type of assay sometimes referred to as a "sandwich assay", immobilized antibodies that specifically react with HIG2 polypeptide are contacted with the biological tissue or fluid sample. Presence of the immobilized HIG2-antibody complex could then be achieved by application of a second, labeled antibody

immunoreactive with either the HIG2 polypeptide or the HIG2-antibody complex. A Western blot type of assay could also be used in an alternative embodiment of the present invention.

If a removed tissue is to be analyzed *in vitro*, typically, degradation of the tissue is preferred prior to testing for the presence of either an mRNA transcript or a gene product. For instance, if detection of polynucleotides is desired, proteolytic degradation is useful (Temsamani et al., Patent No. 5,693,466). Extraction or isolation of proteins or nucleic acids in the sample is also preferred prior to carrying out a diagnostic screen. Numerous methods for the isolation of proteins or nucleic acids from cells or biological fluids are well established in the art.

In a preferred embodiment, a diagnostic evaluation of hypoxia-induced gene expression involves assaying the expression levels of more than one hypoxia-inducible genes at a time. The arrays of the invention are particularly useful for assaying the expression of multiple hypoxia-inducible genes in parallel. The diagnostic detection methods mentioned above in regard to *in vitro* detection would also apply as methods for detecting the presence of polynucleotides and polypeptides in a tissue or a body fluid upon administration of a sample of the tissue or fluid to one of the arrays of the present invention.

Use of the polynucleotide or antibody arrays of the present invention for determining the presence of hypoxia in a tissue of an animal or for evaluating a hypoxia-related condition in a tissue of an animal allows for an unprecedented look at the exact nature and stage of the hypoxic response of a tissue, since the hypoxia-induced expression of a combination of genes is screened at one time. Patterns of expression of hypoxia-inducible (or hypoxia-repressible) genes are complex and highly indicative of hypoxia in a tissue, as demonstrated in the specific examples shown below, Examples 8 and 9. The pattern of expression of hypoxia-inducible genes can therefore be used in a diagnostic or prognostic manner to aid in the treatment of a hypoxia-related condition in an animal.

Information on the pattern of expression of a combination of hypoxia-induced genes can readily be correlated with the aggressiveness of a tumor for instance, thereby providing knowledge critical for establishing the best line of treatment.

The polypeptide arrays of the present invention also can be used to screen  
5 for drugs useful in the treatment of hypoxia-related conditions. These drugs may be drugs which are capable of inhibiting the hypoxic response of a tissue.

For instance, methods of assaying for expression of hypoxia-inducible genes in a tissue in an animal, determining the presence of hypoxia in a tissue in an animal, or evaluating a hypoxia-related condition in a tissue in an animal  
10 comprise first contacting the proteins or messenger RNA of a sample of body fluid or tissue obtained from the animal with an antibody array or polynucleotide array, respectively, of the invention. Tissue or fluid samples from an animal may be contacted directly with an array, and binding of the proteins or mRNA transcripts on the array detected. (The cells in a tissue to be assayed would preferably be  
15 lysed prior to application to the array.) Alternatively, the tissue or fluid sample may be purified to isolate the proteins or mRNA transcripts prior to application to the array. In an alternative embodiment of the method, cDNA is first prepared from the messenger RNA of the sample by reverse transcription and then the cDNA is applied to a polynucleotide array. Once the protein, mRNA or cDNA is  
20 delivered to the array, the method comprises detecting the amount and position of the protein, mRNA or cDNA which remains bound to the array after removal of excess or non-bound protein, mRNA, or cDNA.

Additionally, a method of diagnosing a hypoxia-related condition in an animal may optionally comprise the additional step of correlating the result of the  
25 evaluation of the hypoxia-related condition in the tissue in the animal with an appropriate treatment for the animal. The hypoxia-related condition which may be evaluated, diagnosed or treated by any of the above methods may a condition such



as cancer, ischemia, reperfusion, retinopathy, neonatal distress, preeclampsia, cardiac arrest, or stroke.

Another aspect of the invention provides for a method of treating a tumor. This method involves first determining the presence of hypoxia in a tumor by any of the methods described above (with or without arrays). The method further comprises treating said tumor with any combination of an established form of therapy for cancer such as radiation therapy, chemotherapy, or surgery.

The *HIG1* or *HIG2* polynucleotides or the polynucleotides corresponding to the gene sequences of other hypoxia-inducible gene sequences, such as those listed in Table 1, may be used to attenuate the response of a tissue to hypoxia. These hypoxia-inducible sequences can be targeted within a tissue by the introduction of antisense oligonucleotides, triple-helix probes, catalytic nucleic acids or the like in a manner which inhibits expression of the HIG genes or other hypoxia-inducible genes within the tissue.

Therefore, in one embodiment, the method of attenuating the hypoxic response of tissue comprises inhibiting the expression of a gene selected from the group consisting of *HIG1*, *HIG2*, *annexin V*, *lipocortin 2*, *hnRNP A1*, *Ku autoantigen*, *phosphoribosylpyrophosphate synthetase*, *acetoacetylCoA thiolase*, *ribosomal L7*, *fibroblast growth factor-3*, *EPH receptor ligand*, *plasminogen activator inhibitor-1*, *macrophage migration inhibitory factor*, *fibronectin receptor*, *lysyl hydroxylase-2*, *endothelin-2*, *B-cell translocation gene-1*, *reducing agent and tunicamycin-responsive protein*, *CDC-like kinase-1*, *quiescin*, *growth arrest DNA damage-inducible protein 45*, *DEC1*, *low density lipoprotein receptor related protein*, *hamster hairy gene homologue*, *adipophilin*, *cyclooxygenase-1*, *fructose bisphosphatase*, *creatine transporter*, *fatty acid binding protein*, *lactate dehydrogenase*, *Bcl-2-interacting killer*, *Nip3L/Nix*, and *Pim-1* in said cell. This inhibition of expression of a hypoxia-inducible gene may optionally be achieved by introducing into the cells of said tissue a nucleic acid molecule such as an

antisense oligonucleotide, a triple-helix probe, a deoxyribozyme, or a ribozyme which is specific to the hypoxia-inducible gene.

In an alternative embodiment of the invention, the HIG1 or HIG2 proteins or other expression products of hypoxia-inducible genes may instead be targeted to attenuate the hypoxic response of a tissue. For this purpose, antibodies, antagonists, inhibitors, or proteases that are specific to the expression products of hypoxia-induced genes may be introduced to the tissue.

In one embodiment, a method of attenuating the hypoxic response of a tissue comprises neutralizing a protein selected from the group consisting of HIG1, HIG2, annexin V, lipocortin 2, hnRNP A1, Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3, EPH receptor ligand, plasminogen activator inhibitor-1, macrophage migration inhibitory factor, fibronectin receptor, lysyl hydroxylase-2, endothelin-2, B-cell translocation gene-1, reducing agent and tunicamycin-responsive protein, CDC-like kinase-1, quiescin, growth arrest DNA damage-inducible protein 45, DEC1, low density lipoprotein receptor related protein, hamster hairy gene homologue, adipophilin, cyclooxygenase-1, fructose biphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1. In this method an agent specifically targeting the protein is optionally introduced into the cells of the tissue and can be an antibody, an antagonist, an inhibitor, or a protease.

The methods described above for attenuating the hypoxic response of a tissue may be used to treat a hypoxia-related condition in an animal. For instance, the treatment of a hypoxia-related condition in an animal may be effected by targeting the hypoxia-induced gene sequences of the hypoxic (or potentially hypoxic) tissue via one or more of the techniques known to those skilled in the art. These techniques include, but are not limited, to introduction of antisense oligonucleotides, triple-helix probes, deoxyribozymes, or ribozymes into the

subject's tissue of concern. In a preferred embodiment, the animal to be treated is a human. The hypoxia-related condition towards which this treatment may be directed is ischemia, stroke, heart attack, neonatal distress, retinopathy, or any other disease condition in which hypoxia plays a significant role. In another  
5 embodiment, the hypoxia-related condition to be treated is cancer and the tissue is a tumor. The disclosed treatment of the tumor may be coupled with any combination of other cancer therapies such as radiation therapy, chemotherapy, or surgery.

Similarly, treatment of the hypoxia-related conditions may also be achieved  
10 by neutralizing the protein expression products of hypoxia-inducible genes, as described above. In accordance with this method, antibodies, antagonists, inhibitors, proteases, or the like which target and neutralize HIG1 and HIG2 polypeptides may be introduced into the animal, preferably human, containing the tissue to be treated.

15 The protein expression products of the genes which have been newly identified as being hypoxia-inducible may be used to identify or screen for drugs, such as inhibitors, useful in the treatment of hypoxia-related conditions. For instance, small molecule drug candidates or peptides may be tested against the any of the proteins of HIG1, HIG2, annexin V, lipocortin 2, hnRNP A1, Ku  
20 autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3, EPH receptor ligand, plasminogen activator inhibitor-1, macrophage migration inhibitory factor, fibronectin receptor, lysyl hydroxylase-2, endothelin-2, B-cell translocation gene-1, reducing agent and tunicamycin-responsive protein, CDC-like kinase-1, quiescin, growth arrest DNA  
25 damage-inducible protein 45, DEC1, low density lipoprotein receptor related protein, hamster hairy gene homologue, adipophilin, cyclooxygenase-1, fructose biphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, or Pim-1 to see if inactivation

of the enzymatic activity or prevention of crucial binding activity of the hypoxia-induced protein occurs. Combinatorial libraries of small molecules or libraries of peptides such as those produced by phage display may alternatively be screen against one of the hypoxia-induced proteins described herein.

5       The expression of some gene products induced by hypoxia can be helpful in protecting cells from damage or death. Thus, this invention also provides for methods of enhancing the hypoxic response of a tissue and thereby and treating hypoxic tissue (or potentially hypoxic tissue). The method comprises introducing an expression vector into the tissue and allowing for expression of the coding  
10       sequence on the vector to take place. The coding sequence of the expression vector comprises the sequence of at least one of the genes *HIG1*, *HIG2*, *annexin V*, *lipocortin 2*, *hnRNP A1*, *Ku autoantigen*, *phosphoribosylpyrophosphate synthetase*, *acetoacetylCoA thiolase*, *ribosomal L7*, *fibroblast growth factor-3*, *EPH receptor ligand*, *plasminogen activator inhibitor-1*, *macrophage migration*  
15       *inhibitory factor*, *fibronectin receptor*, *lysyl hydroxylase-2*, *endothelin-2*, *B-cell translocation gene-1*, *reducing agent and tunicamycin-responsive protein*, *CDC-like kinase-1*, *quiescin*, *growth arrest DNA damage-inducible protein 45*, *DEC1*, *low density lipoprotein receptor related protein*, *hamster hairy gene homologue*, *adipophilin*, *cyclooxygenase-1*, *fructose biphosphatase*, *creatine transporter*,  
20       *fatty acid binding protein*, *lactate dehydrogenase*, *Bcl-2-interacting killer*, *Nip3L/Nix*, or *Pim-1*. Expression of the vector's hypoxia-inducible gene within the tissue should occur at a level which is higher than would occur in the absence of the expression vector. Depending on the particular use, the coding sequence of the expression vector may be operably linked to its native promoter, another  
25       hypoxia-inducible promoter, or a constitutive promoter.

Alternatively, the proteins of the hypoxia-inducible genes may be introduced into the tissue directly to enhance the hypoxic response of the tissue and for treatment of hypoxia. Delivery of the proteins may be achieved through

the use of liposomes, hydrogels, controlled-release polymers, or any of the other vehicles known in the art to be useful for the delivery of polypeptides as drugs.

e) Methods for Identifying Stress-Inducible Genes

To facilitate efforts to identify hypoxia-inducible genes, we modified and improved a PCR subtraction method known as Representational Difference Analysis (RDA) (see specific example, Example 1, below, and Figures 7 and 8). The RDA method has been used to distinguish differences between genomic DNA from two related, but different sources (Wigler et al., Patent No. 5,436,142). The RDA technique involves selectively amplifying via polymerase chain reaction only fragments of those sequences contained within one DNA sample, but not the other. The selectivity of the amplification step used in this method is not precise, but is sufficient to detect differences in the genomes of two human individuals.

The present invention provides for methods of identifying both stress-inducible and stress-repressible genes. The methods identify differences between mRNA from cell populations exposed to different stress conditions. A representative protocol for the identification of stress-inducible genes is outlined in detail in a specific example below (Example 1).

The method for identifying stress-inducible or stress-repressible genes and fragments of genes involves first subjecting one of two populations of cells to stress prior to preparation of two cDNA libraries from the mRNA libraries of the two populations. Protocols for the generation of cDNA libraries through reverse transcription of mRNA sequences are well known in the art and kits for doing so are commercially available (from Gibco BRL, for instance). In a preferred embodiment of the method, the cDNAs are synthesized by using a mixture of oligo-dT primers containing equal proportions of oligomers having a G, A, or C residue at the 3'-end ("indexed" or "registered" primers). This approach ensures that a given primer will hybridize at the start of a polyA tail sequence of an mRNA rather than randomly within the sequence. These oligo-dT primers also

have a defined DNA sequence (20 to 24 base pairs in length) that is incorporated into each cDNA fragment. This tag permits the use of two PCR primers to specifically amplify the 3'-end of each cDNA. The two cDNA libraries are digested separately with restriction enzymes and then linker sequences are ligated to the ends of the digested cDNA fragments, as shown in Fig. 7. Restriction digests and ligation of linkers may be performed in any manner known to those skilled in the art. Some examples of such methods may be found in Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual, 2nd. ed*, Cold Spring Harbor Laboratory Press, herein incorporated by reference.

The cDNA library from one of the two cell populations is amplified with tagged oligonucleotide primers by means of the polymerase chain reaction (PCR). In a preferred embodiment, the "tag" on the oligonucleotide primers is biotin. However, any chemical or biological moiety which provides a means of selection or isolation of the tagged entity (by affinity chromatography, for instance) is suitable as a tag. In the preferred embodiment, use of biotin as a tag allows for removal of the tagged sequences on a streptavidin resin. In an alternative embodiment, however, oligonucleotides bearing a thiol group, for example, may instead be used as the tagged primer, since oligonucleotides with attached thiol groups can be retained on a variety of affinity resins, such as thiopropyl sepharose columns or mercurial resins. The cDNA library PCR-amplified with tagged primers is referred to herein as "driver" cDNA.

The cDNA library from the stressed cells is amplified with normal, non-tagged, oligonucleotide primers in a separate polymerase chain reaction. The cDNA PCR-amplified in this manner is referred to herein as "tester" cDNA.

The non-tagged, amplified, tester cDNA is heated and then reannealed in the presence of a large excess (typically about 5- to about 100-fold) of the tagged, amplified, driver cDNA. See Fig. 8. Next, those DNA strands which either are themselves tagged or are duplexed with tagged DNA are removed from the

mixture. This removal is typically done via exposure of the mixture of DNA strands to a resin or matrix which has affinity for the tag used on the primers earlier. In a preferred embodiment, magnetic beads coated with streptavidin are used. Other resins, such as streptavidin agarose could be used in conjunction with  
5 a biotin tag. Tagged single-stranded or duplex cDNA will be retained on the affinity resin, and the non-tagged species, which are not retained, can be found in the flowthrough or supernatant. In this technique, the cDNA from the non-stressed cell population is "subtracted" from the cDNA of the stressed cell population. The remaining, non-tagged cDNA library is said to be "enriched".

10 The remaining, non-tagged cDNA sequences are then again amplified by means of the polymerase chain reaction with non-tagged primers.

After amplification of the remaining non-tagged cDNA sequences, the non-tagged cDNA library is again heated and reannealed in the presence of a large excess (typically about 5- to about 100-fold) of the original tagged cDNA library.

15 Removal of all tagged DNA molecules and reamplification of remaining tagged sequences again follows. The combination of steps involving heating and reannealing, removed tagged molecules, and reamplifying remaining, non-tagged molecules constitutes one round. The methods of the present invention involve repeating the rounds from zero to many times. In a preferred embodiment, the  
20 method involves a total of approximately 3 to 5 rounds.

In a particularly preferred embodiment, the method involves performing the steps as described above in parallel with a second set of steps in which the cDNA library from the stressed population of cells is instead subtracted from the cDNA library from the non-stressed population. This means that in the second set of  
25 steps, the cDNA library from the stressed cell population is amplified with tagged primers and the cDNA library from the non-stressed cell population is amplified with non-tagged primers. The original cDNA of the stressed cell population is repeatedly subtracted from the cDNA of the non-stressed cell population, and

separately, the original cDNA of the non-stressed cell population is repeatedly subtracted from the stressed cell population.

In the final round of the preferred embodiment of the method, one of the two enriched cDNA libraries obtained from the two sets of steps is subtracted  
5 from the other enriched cDNA library. Which enriched library is subtracted from which is entirely dependent upon whether stress-inducible or stress-repressible sequences are sought. If stress-inducible sequences are sought, the enriched, non-stressed cDNA library is subtracted from the enriched, stressed, cDNA library. If stress-repressible sequences are sought, the enriched, stressed-cell cDNA library is  
10 subtracted from the enriched non-stressed-cell cDNA library.

The final subtraction step of one enriched library against another is beneficial since the initial subtraction rounds of the procedure tend to remove only the cDNAs that are in common and present at high frequency in the two populations, because cDNA fragments derived from rare messages will initially be  
15 present at such low concentrations that they might not find a complementary strand during the hybridization step. After the major sequences in common are removed by subtraction, the rare sequences will begin to increase in concentration so that they can then be effectively subtracted. After multiple cycles of subtraction are performed, the rarest sequences from both conditions are enriched  
20 in the libraries, and subtraction of one enriched library from another yields an effective isolation of either stress-inducible or stress-repressible genes.

After the desired number of rounds of subtraction have been completed, the enriched cDNA library may be cloned and sequenced using any one of the multitude of techniques known to those skilled in the art. A particularly  
25 convenient method of inserting PCR-amplified DNA strands into vectors suitable for cloning and sequencing, known as "T-A cloning", is commercially available from companies such as Invitrogen and Novagen. Other alternative methods can



be found in *Molecular Cloning: A Laboratory Manual, 2nd. ed, Vol. 1-3*, eds. Sambrook et al., Cold Spring Harbor Laboratory Press (1989).

In one embodiment, the stress to which one of the two cell populations is exposed is hypoxia. The method may also be applied to the investigation of  
5 responses to other stresses, such as ionizing radiation, heat, glucose starvation, hypothermia, or pH change. Alternatively, the response to a stress such as a toxin or a drug may be investigated by employment of the disclosed method.

f) Examples

10 The following specific examples are intended to illustrate the invention and should not be construed as limiting the scope of the claims.

Example 1. Generation of Enrichment cDNA Libraries

Normal human cervical epithelial cells stably immortalized with the human papillomavirus E6 and E7 oncoproteins (HCE.E6.E7) served as the starting  
15 material for the construction of a cDNA library enriched by representational difference analysis (RDA). HCE.E6E7 were cultured in synthetic medium PFMR-4A (Kim et al. (1997) *Cancer Res.* 57:4200-4).

A total of 5  $\mu$ g of poly A<sup>+</sup> mRNA from both HCE.E6E7 cells cultured under hypoxic (5% CO<sub>2</sub>/5% H<sub>2</sub>/90% N<sub>2</sub> for 16 hours at 37°C) conditions and  
20 HCE.E6E7 cells cultured under aerobic (5% CO<sub>2</sub> / 20% O<sub>2</sub> / 75% N<sub>2</sub> at 37°C) conditions were used to generate double-stranded cDNA preparations by using the Gibco BRL cDNA Synthesis System.

Hypoxic conditions were generated by the use of an anaerobic chamber (Sheldon Laboratories, Cornelius OR) that is flushed with a gas mixture of 90%  
25 N<sub>2</sub>, 5% CO<sub>2</sub> and 5% H<sub>2</sub>. Any oxygen that was introduced into the chamber was consumed over a catalyst with hydrogen. A monitoring oxygen electrode was used to confirm an environment of 0.05% oxygen or less during experimentation.

One-fifth of the cDNA product (approximately 1-1.5 µg) from the hypoxic or oxic cells was digested with 20 units of the *Nla* III restriction enzyme, 50 mM potassium acetate, 1 mM DTT, and 100 µg/ml bovine serum albumin for 60 min at 37°C. The reaction mixture was extracted with phenol and chloroform,  
5 precipitated with ethanol, redissolved in 10µL of water and lyophilized. Ethidium agarose gel electrophoresis was used to verify that the cleavage was successful.

For each pair of cDNAs used for the RDA procedure (i.e. the “test” and the “driver”), two different DNA linkers were attached by ligation to the *Nla* III cleaved ends. The 3’-end of the linker sequence opposite the ligation site was  
10 terminated with an amine so that it cannot be used as an acceptor or donor for a ligase. The linker oligonucleotides used were as follows (where “X” denotes the amino-terminated residue at the 3’-end of the shorter of the two strands):

5’-TTTTACCAGCTTATTCAATTCGGTCCTCTCGCACAGGATGCATG-3’

15 (SEQ ID NO:11)

XATGGTCGAATAAGTTAAGCCAGGAGAGCGTGTCTAC-5’ (SEQ ID NO:12)

5’-TTTTTGTAGACATTCTAGTATCTCGTCAAGTCGGAAGGATGCATG-3’ (SEQ ID NO:13)

20 XAACATCTGTAAGATCATAGAGCAGTTCAGCCTTCCTAC-5’ (SEQ ID NO:14)

(The linker pair of SEQ ID NO:13 and SEQ ID NO:14 was used for the hypoxically incubated cell cDNAs.) The two separate linker strands were  
25 dissolved in 10 mM Tris-HCl (pH 7.6), 10 mM MgCl<sub>2</sub> buffer (10 µM of each oligomer), then heat-denatured and slowly cooled to room temperature before use in a ligation reaction.

Next, 1 µg of the *Nla* III cleaved cDNA was ligated in a 100 µL volume of 1 µM double-stranded linker, 5% polyethylene glycol, 50 mM Tris-HCl (pH 7.6), 10 mM MgCl<sub>2</sub>, 1 mM ATP, and 1 mM DTT at 16°C for 3 h. Since the linkers used to ligate to the cDNA fragments do not have a phosphate at the 3'-end of the *Nla* III overhang, the resulting ligation products have a single-stranded nick. Performing the reaction in this way had the advantage of preventing self-ligation of the linkers. The excess linkers were removed by gel filtration through a spin-column containing Sephacryl S-300HR. The linker-ligated cDNA fragments were collected in the microfuge tube while the excess unligated linkers were trapped in the Sephacryl with other low molecular-weight components. The gel-filtered, linker-ligated cDNA fragments were then lyophilized to dryness.

The linker-ligated cDNA fragments were amplified by a single-primer PCR technique. Again, if the preparation was to be used as the driver cDNA, it was amplified by using PCR primers with a biotin residue at the 5'-end. If the preparation was to be used as the test cDNA from which the driver is used to subtract sequences, then it was amplified by using untagged primers.

The ligated cDNA (0.1 µg aliquot) was amplified in a standard PCR buffer containing 1 µM primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.01% gelatin. Before PCR amplification, the nicked PCR template had to be repaired by TAQ polymerase during a 5-min extension reaction at 72°C. After this initial incubation, a standard PCR reaction of 35 cycles (94°C, 30s; 56°C, 30s; 72°C, 60s) was performed in a Perkin Elmer DNA Thermal Cycler. The oligonucleotide primers used in the amplification step were as follows:

- 5'-CCAGCTTATTCAATTCGGTCC-3' (SEQ ID NO:15)  
5'-GTAGACATTCTAGTATCTCGT-3' (SEQ ID NO:16)

(SEQ ID NO:16 was the primer used to amplify cDNA from the hypoxically incubated cells.) The entire PCR reaction was passed through a 1 ml Sephacryl spin column as described above to remove salts, dNTPs, and excess primers. The yield of the amplification was determined by ethidium agarose gel electrophoresis.

5 The product appeared as expected as a smear of DNA fragments ranging from 100 to 2,000 base pairs (bp) in size.

The first round of subtraction was performed by mixing 3  $\mu$ g of the biotinylated driver cDNA with 0.1  $\mu$ g of the test cDNA. The mixture was lyophilized in a 0.5 mL microfuge tube and carefully redissolved in 2  $\mu$ L of 10 mM HEPES (pH 7.5), 10 mM EDTA, 1.5 mM NaCl, and 2% sodium dodecyl sulfate (SDS). This very small amount of solution was overlaid with 50  $\mu$ L of mineral oil to prevent evaporation, and the tube was place in the thermal cyclor and heated at 95°C for 10 min. It was then slowly cooled to 68°C over a period of 1 h, after which the incubation at 68°C was continued for a further 4 hours. At 15 the end of the incubation, 100  $\mu$ L of the same HEPES buffer at 68°C was added to the tube. The diluted solution was then cooled to room temperature and the mineral oil removed.

The biotinlyated cDNAs and any hybridized sequences were removed by mixing the diluted solution with a 100  $\mu$ L slurry containing 1 mg of M-280 20 Streptavidin Dynabeads (Dyna) in the same incubation buffer. The incubation was continued at room temperature for 30 min with slow tumbling. The beads were then pelleted to the bottom of the tube by using a magnet and the supernatant was removed and desalted by passing through a 1 mL Sephacryl spin column as described above. The cDNA solution was then lyophilized and redissolved in 10 25  $\mu$ L of water.

The small amount of cDNA remaining after subtraction was reamplified by PCR using the same primers. A single-stranded binding protein was added to the PCR reaction mixture used to reamplify the subtracted cDNA fragments: 1  $\mu$ L

(one-tenth volume) of the subtracted cDNA preparation was placed in 100  $\mu$ L of PCR buffer containing 1  $\mu$ g of *Escherchia Coli* single-stranded binding protein (Perfect Match™, Stratagene). The cDNA was amplified during 25 PCR cycles (94°C, 30 s; 54°C, 30 s; 72°C, 60 s), and the product was analyzed by ethidium  
5 agarose gel electrophoresis. The appearance of this reamplified cDNA was similar to that of the initial material described above.

Multiple rounds of subtraction were performed. The subtraction libraries were prepared in parallel, so that the library enriched for sequences expressed under hypoxic conditions was prepared at the same time as the library enriched for  
10 sequences expressed under normoxic conditions. In each case, the driver used for the initial rounds of subtraction was the original set of cDNA fragments. After three rounds of subtraction, the enriched library prepared in parallel was used as the driver for the fourth round. In this way, the rarest sequences from both conditions were enriched in the final library. For instance, to obtain hypoxically  
15 induced sequences in this final round, the cDNA library enriched for sequences expressed under normoxic conditions served as the driver library and the cDNA library enriched for sequences expressed under hypoxic conditions served as the test library.

#### Example 2. Sequence Identification and Northern Blot Analysis of Significant 20 Isolated Expressed Sequence Tags (ESTs).

Several hundred cDNA fragments were sequenced from each of the two enrichment libraries produced by the subtraction protocol of Example 1 from HCE.E6E7 cells cultured under hypoxic and aerobic conditions. Four rounds of RDA subtraction of the oxic cDNAs from the hypoxic cDNAs generated a  
25 population of fragments in one of the enrichment libraries representing genes that theoretically are induced by hypoxic treatment. Five hundred randomly chosen clones from the cDNA library were partially sequenced. The obtained sequences

were analyzed by NCBI-blast to determine the frequency of each of the genes/ESTs in the enriched population and to identify whether the isolated, hypoxia-induced ESTs corresponded to previously identified genes or ESTs.

The frequencies of EST sequences among the 500 randomly chosen cDNA fragments obtained from the cDNA library enriched for sequences expressed under hypoxic conditions (after all four rounds of subtraction) is shown in Table 2, below. The two most frequently occurring ESTs, the HIG1 EST and the HIG2 EST, corresponded to no known genes. Because these most frequently repeated clones were unknown, the full-length cDNAs representing HIG1 and HIG2 were isolated (see Example 3, below).

All the ESTs present in the clones of each library that were represented more than one time and that did not contain a highly repetitive element were tested by Northern blot for induction by hypoxia in Siha cervical carcinoma cells (and/or in HCE.E6E7 cells). Selected probes representing ESTs found more than once were applied to Northern blots of total RNA from cell cultures harvested following different aerobic and hypoxic exposures to verify hypoxia inducibility or repressibility. For instance, the northern blot assays were used to confirm that,  $\alpha$ -tubulin mRNA, detected in the HCE.E6E7 aerobic enrichment library, decreased in response to hypoxia in HCE.E6E7 cells, whereas mRNA corresponding to the HIG2 EST, found in the hypoxic enrichment library, strongly increased under the same hypoxic conditions.

**Table 2. Tags isolated from the cDNA library following four rounds of RDA subtraction of oxid cDNAs from hypoxic cDNAs.**

| # of hits | tag name | gene to which EST corresponds | accession # of gene | response to hypoxia* | comment    |
|-----------|----------|-------------------------------|---------------------|----------------------|------------|
| 106       | HIG1     | HIG1                          |                     | induced              | novel gene |
| 98        | HIG2     | HIG2                          |                     | induced              | novel gene |

|    |       |                            |          |                |                             |
|----|-------|----------------------------|----------|----------------|-----------------------------|
| 48 | HIG3  | GAPDH                      | J04038   | induced        | inducibility prev.<br>known |
| 11 | HIG4  | HNRNP                      | X12671   | induced        |                             |
| 11 | HIG5  | Annexin V                  | U01691   | induced        |                             |
| 8  | HIG6  | AcetoacetylCoA<br>thiolase | S70154   | induced**      |                             |
| 7  | HIG7  | Tissue Factor              | X67698   | induced        | inducibility prev.<br>known |
| 7  |       | 5-2A bp                    | X76388   | not induced    |                             |
| 6  |       | unknown gene               | clone 68 | no signal      |                             |
| 5  |       | Alu-like                   |          | not determined |                             |
| 5  | HIG8  | Lipocortin 2               | M14043   | induced        |                             |
| 5  | HIG9  | Ribosomal L7               | X57959   | induced        |                             |
| 4  |       | unknown gene               | clone 24 | not induced    |                             |
| 3  |       | Vacuolar ATPase            | X71490   | no signal      |                             |
| 3  | HIG10 | PRPP synthase              | D00860   | induced        |                             |
| 3  |       | Alu-like                   |          | not determined |                             |
| 2  |       | RNA pol1 40Kd<br>subunit   | AF047441 | not induced    |                             |
| 2  | HIG11 | thioredoxin                | X77584   | induced        |                             |
| 2  |       | hSRP1(nuc loc)             | U28386   | not induced    |                             |
| 2  | HIG12 | Ku(70)                     | J04611   | induced        |                             |
| 2  |       | Sm protein F               | X85382   | not induced    |                             |
| 1  |       | 168 different clones       |          |                |                             |

\* as determined by Northern blot

\*\* minor 4.2kB acetoacetylCoA thiolase message only is induced

The procedure for the Northern blot assay was essentially as follows. Total RNA was isolated with Trizol (Gibco BRL) following the directions of the manufacturer. 5-10 µg of total RNA was denatured with glyoxal and size fractionated on a 1% agarose phosphate gel. The gel was capillary transferred to Hybond nylon (Schleicher and Shuell) and UV cross-linked. Probes were radiolabeled by random priming of gel-purified full length HIG1, or a fragment of HIG2 containing only the coding sequence in a StuI fragment (Rediprime, Amersham). Hybridization was carried out in 0.5 M Na<sub>2</sub>HPO<sub>4</sub>, 7% SDS, 1 mM EDTA at 56°C for HIG1 and 65°C for HIG2, washed to 0.2-0.5 x SSC at 56°C or 65°C, exposed to a phosphorimager plate, and visualized on a Storm 860 phosphorimager (Molecular Dynamics).

The hypoxia-inducibility of ESTs as determined by Northern blot is summarized in Table 2, above. The HIG1 and HIG2 sequences both demonstrated hypoxia-inducibility in the Northern blot assay.

Northern blots of total RNA from various aerobic and hypoxic human cells [HCE.E6E7s; SiHa cervical squamous carcinoma, MCF-7 breast carcinoma, H1299 lung carcinoma, Hct116 colonic carcinoma cells; human cervical fibroblasts (HCFs) and HCF.E6E7s] probed for HIG2 expression demonstrated the following: (1) the gene is expressed as a single 1.5 kb transcript (the original EST cross-hybridizes with unknown 1.6- and 4-kb transcripts in HCE.E6E7s); (2) HIG2 mRNA increases from undetectable in 21% O<sub>2</sub> (air) to abundant in 0.02% O<sub>2</sub> in HCE.E6E7, SiHa, and MCF-7 cells after 6 h of hypoxia; (3) HIG2 is moderately expressed in H1299 and Hct116 cells after 6 h of hypoxia; (4) there is no detectable HIG2 mRNA in HCFs and HCF.E6E7s; (5) in SiHa cells, HIG2 remains elevated for 48 h of hypoxia but decreases moderately by 72 h of exposure; and (6) no HIG2 induction is found in SiHa cells 6 h and 24 h after treatment with UV-C (20 J/m<sup>2</sup>), γ-irradiation (6 Gy), MMS (100 µg/mL for 1 h), serum deprivation (0.1%), or glucose starvation (4%, <1 mM); (7) HIG2



expression is extinguished after exposure of hypoxic cells to 2 hours of reoxygenation.

The hypoxia inducibility of *HIG1* has been found to range between about 2-fold and about 5-fold across a variety of different human cell lines studied. The  
5 hypoxia-inducibility of *HIG2* ranges between about 10- and about 20-fold across the various human cell lines studied. (See also Example 4, below).

In addition to the novel genes *HIG1* and *HIG2*, several known genes identified by the subtraction method in Example 1 were confirmed by Northern blots to be hypoxia inducible. These genes are also listed in Table 2. ESTs  
10 corresponding to the genes of annexin V, lipocortin 2, hnRNP A1, Ku (70) autoantigen, glyceraldehyde-3-phosphate dehydrogenase, ribosomal L7, acetoacetylCoA thiolase, and PRPP synthetase were identified by multiple hits in the hypoxia screen. All of these previously known genes were confirmed to be hypoxia-inducible by Northern blot.

15 It should be noted that although acetoacetyl CoA thiolase sequence tag is listed as induced, the reported, major RNA (1.8 kb) for the gene does not change. However, there is a larger, hybridizing, RNA species (4.2 kb) that is induced after 24-48 h hypoxia (data not shown).

ESTs corresponding to glyceraldehyde 3-phosphate dehydrogenase  
20 (GAPDH) were especially prevalent amongst the cDNA clones. The hypoxia-induced expression of glyceraldehyde-3-phosphate dehydrogenase had been previously identified only in normal, non-transformed cells.

### Example 3. Isolation and Analysis of Full-Length *HIG1* and *HIG2* cDNA 25 Sequence

The *HIG2* EST (142 bp) was used to probe a conventional cDNA library constructed from mRNA isolated from SiHa cells exposed to 16 h hypoxia to obtain the full-length cDNA clone *HIG2*. This library was probed with

radiolabelled HIG2 tag using conventional methods. Full length *HIG1* was isolated by first identifying overlapping ESTs from the NCBI human EST database, until a full length sequence was generated (1.35 kb). PCR primers were then synthesized corresponding 5' and 3' UTRs in order to amplify the complete  
5 sequence using RT-PCR of SiHa RNA isolated after a 16 h hypoxia treatment. The full-length *HIG1* cDNA was then cloned and sequenced to confirm the predicted sequence.

The full-length cDNA sequence of *HIG1* is shown in Figure 1A. The full-length cDNA sequence of *HIG2* is shown in Figure 2A. The translations of the  
10 putative open reading frames from *HIG1* and *HIG2* are listed in Figure 1B and 2B, respectively, and both encode small peptides (95 and 64 aa residues respectively) without obvious functional motifs.

Example 4. Hypoxic induction of *HIG1* and *HIG2* in cervical cancer cell lines.

Because *HIG1* and *HIG2* represent two novel genes whose functions are unknown, these genes were investigated in more detail. The expression of *HIG1* and *HIG2* was examined in a series of human cervical cancer cell lines (SiHa, CaSki and C33a) under oxic and hypoxic conditions *in vitro*. (The cell lines SiHa, CaSki and C33a were obtained from the ATCC and were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI1640 supplemented with 10% fetal bovine serum.) Although *HIG1* is induced moderately within 2 hours of hypoxia in all the cell lines tested, it remains elevated only in the SiHa cells. *HIG2* is more consistently induced from low basal levels in all the cervical cancer cells tested. The major *HIG2* mRNA species is 1.4 kb in length, but there are two other mRNA species of minor abundance (8.0 and 9.0 kb) that are induced with identical kinetics to the major species.

Example 5. Hypoxic induction of *HIG1* and *HIG2* in tumor xenografts.

The hypoxic induction of *HIG1* and *HIG2* *in vivo* was also tested in tumor xenografts generated from the C33a cell line by Northern blot analysis of total tumor RNA. Gene expression in untreated xenografts was compared to that in xenografts that were made hypoxic by treatment of the host animal with flavone acetic acid (FAA) 24 hours prior to explantation and RNA isolation. To generate tumor xenografts,  $2.5-5 \times 10^6$  cells were injected subcutaneously into the flank of scid mice and allowed to grow into tumors that reached 1-2 cm in diameter before harvest. FAA (Lipha Chemical, NY) was injected IP into the animals at 200 mg/kg in 5% sodium bicarbonate 24 hours prior to tumor harvest. FAA treatment resulted in increased tumor hypoxia as measured by ependorff electrode and increased *HIG1* and *HIG2* expression by 1.2 and 2.4 fold respectively. The moderate level of *HIG1* induction *in vivo* is not unexpected, due to the *in vitro* data. The portion of the human gene used for a probe in these experiments has

low homology with mouse RNA and under the conditions used, did not cross-hybridize.

Example 6. Specificity of the induction of *HIG1* and *HIG2*.

5        We next investigated whether *HIG1* and *HIG2* induction is unique to hypoxic stress, or if it is elicited by other tumor microenvironment stresses such as glucose deprivation, serum starvation, or by genotoxic stresses such as UV or ionizing radiation. We also tested the hypoxia-mimetic, iron-chelating compound desferoxamine that has been shown to induce expression from HIF-1 responsive  
10    genes. For stress treatments, cells were plated overnight and then treated the next day with either 256 nm UV at 1.2 J/m<sup>2</sup>/sec, or gamma irradiation from <sup>137</sup>Cs source at 3.8 Gy/min. Glucose and serum deprivation experiments were performed by washing the cells three times in phosphate-buffered saline (PBS) and replacing the indicated media (glucose free RPMI with dialyzed serum, or  
15    0.1% FBS RPMI).

Northern blot analyses was performed on RNA isolated from C33a cells exposed to these stresses. *HIG1* was poorly responsive to hypoxic stress over this timecourse, but strongly induced by glucose deprivation. *HIG2* was induced strongly by hypoxia, the hypoxia-mimetic stress desferoxamine (DFO), and  
20    glucose deprivation. UV light seemed to have little effect upon either *HIG1* or *HIG2* expression. In contrast, while ionizing radiation did not change *HIG1* expression levels, it did result in a moderate 2.5 fold induction of *HIG2* by 24 hours. There were similarities in the pattern of stress responsiveness of *HIG2* and that of the HIF-responsive VEGF gene, suggesting that HIF-1 may be important in  
25    *HIG2* expression.

Example 7. Identification of *HIG1* and *HIG2* sequences from non-human species.

A search of the NCBI-dbEST database for fragments of genes from other species that might represent evolutionarily conserved orthologues identified overlapping mouse EST fragments that encode for similar peptides to the human version of *HIG1* and *HIG2*. The murine *HIG1* and *HIG2* orthologues are shown  
5 in Figures 3A and 5A, respectively. These mouse genes code for predicted peptides (Figures 3B and 5B, respectively) with 84% and 76% identity to the human peptides respectively. There also existed a cDNA cloned from fish (*seriola quinqueradiata*) in the database that coded for a *HIG1* orthologue (Figure 4A and 4B). A sequence comparison of the *HIG1* homologues is shown in Figure  
10 6A. A sequence comparison of the *HIG2* homologues is shown in Figure 6B.

We confirmed the existence of murine *HIG1* and *HIG2* by cloning the presumed genes and assaying for their expression. We designed oligonucleotide primers corresponding to sequences in the 5' and 3' untranslated regions that would amplify these genes. We were able to make primers that amplified the  
15 entire murine *HIG2* cDNA, but were only able to make primers that would amplify the coding sequence for murine *HIG1*:

mHIG1 forward primer (SEQ ID NO:17):

5'-CCGATCTAGAGGAAGGGACCCCGCGTCTCGGA-3'

20 mHIG1 reverse primer (SEQ ID NO:18):

5'-GGCGCTCGAGTCTAAACCCACATGTTATTTATTG-3'

mHIG2 forward primer (SEQ ID NO:19):

5'-CCTTACTCCTGCACGACCTGG-3'

mHIG2 reverse primer (SEQ ID NO:20):

25 5'-GGCGCTCGAGCACATGTGCATTACACTGGAGA-3'

These primers were then used to amplify the coding sequences of *HIG1* OR *HIG2* from reverse-transcribed RNA isolated from the murine squamous cell tumor cell

line SCCVII (cultured in DMEM supplemented with 10% FBS). The amplified fragments were cloned and sequenced, confirming the predicted sequence.

The cloned genes were then used as probes for Northern blot analysis of RNA isolated from SCCVII cells. Both *mHIG1* (murine *HIG1*) and *mHIG2* (murine *HIG2*) have hypoxia-inducible species of RNA by this analysis. Murine *HIG1* has two major RNA species that strongly hybridize to the probe, at approximately 1.2-1.4 kb in length. The larger message is modestly induced, while the smaller message is strongly induced to approximately 5 fold by a 12h exposure to hypoxia. Murine *HIG2* also has two RNA species at approximately 1.4 and 2.2 kb. Both the murine *HIG2* mRNAs seem to be mildly hypoxia-inducible with 2-3 fold induction by 6-12 hours. For comparison, the same blot was probed with vascular endothelial growth factor (VEGF) and this message shows an approximately 5-fold induction by 6h.

Example 8. Analysis of Gene Expression under Hypoxia using Gene Discovery Arrays (GDA).

Nylon filters containing GDA arrays were purchased from Genome Systems (St Louis, MO) that have affixed to them nucleic acids that were originally characterized by the I.M.A.G.E. consortium (LLNL). This array represents 18,394 cDNA clones that have been categorized as either known genes or ESTs (expressed sequence tags) isolated by the consortium. This filter was used to quantitatively determine the mRNA expression levels of all these arrayed cDNAs in SIHA tumor cells both under oxic conditions and hypoxic conditions (18 hrs, <0.2 %). Messenger RNA was isolated from control and hypoxic SIHA cells and cDNA probe was generated using MoML reverse transcriptase. 2 µg mRNA was incubated with 500 ng of oligonucleotide primer (T)<sub>18</sub> NM (N=A/C/G, M=A/C/G/T) in the presence of reaction buffer, 4mM dATP, 4mM dGTP, 4 mM dTTP and 4mM alpha [33]P dCTP and 200U reverse transcriptase.

The radioactively labeled first strand cDNA that was produced from this reaction was then used to probe the respective filter. The filters were then exposed to a phosphorimager plate, the image collected and digitized for analysis, and the relative counts on each cDNA were quantitated and compared using GDA analysis software. The results are shown in Table 3 for the 500 genes or ESTs with the greatest level of hypoxic induction and in Table 4 for the 500 genes or ESTs with the greatest level of hypoxic repression.

**Table 3. Genes (identified by Genbank Accession Number) whose expression was induced in hypoxic cells, shown with the ratio of their expression in hypoxic cells over their expression in oxic cells.**

| ratio | Acc. No. | ratio | Acc. No. | ratio | Acc. No. | ratio | Acc. No. |
|-------|----------|-------|----------|-------|----------|-------|----------|
| 5.18  | AA069408 | 7.013 | AA134027 | 4.027 | AA155910 | 3.372 | AA037436 |
| 7.528 | T48883   | 5.236 | N35559   | 5.343 | T87461   | 2.025 | H22698   |
| 9.999 | N58711   | 5.753 | R63553   | 3.478 | W24548   | 5.454 | W07146   |
| 5.678 | H04904   | 6.525 | N27733   | 5.699 | T48772   | 5.209 | AA101069 |
| 6.453 | H82707   | 3.146 | N94304   | 4.095 | N94916   | 4.599 | W24109   |
| 7.825 | W56465   | 4.218 | H70730   | 6.52  | H14897   | 4.996 | R17409   |
| 9.999 | N31409   | 1.659 | AA053856 | 1.159 | R34659   | 4.932 | W17090   |
| 9.999 | H19264   | 8.836 | W05763   | 2.492 | H93923   | 5.391 | R24601   |
| 3.626 | R73213   | 3.394 | R54524   | 5.054 | N28535   | 5.189 | R26954   |
| 9.999 | R08251   | 8.246 | W15599   | 2.08  | AA069499 | 2.12  | AA187216 |
| 9.999 | H91612   | 4.216 | R09918   | 5.021 | W38635   | 3.852 | H86677   |
| 9.999 | AA196038 | 9.238 | AA005185 | 8.59  | T54127   | 4.653 | H67329   |
| 9.999 | W55913   | 4.654 | T95404   | 4.289 | R26319   | 4.993 | W19173   |
| 5.143 | R94248   | 9.262 | R19946   | 6.048 | W07082   | 5.641 | W06851   |
| 9.999 | R85589   | 4.308 | AA068998 | 3.12  | AA194330 | 6.186 | W00378   |
| 2.9   | H50204   | 5.203 | N47831   | 4.594 | N31417   | 4.458 | AA204792 |

|       |          |       |          |       |          |       |          |
|-------|----------|-------|----------|-------|----------|-------|----------|
| 4.333 | H52973   | 4.175 | AA151009 | 2.424 | AA069173 | 4.64  | W48584   |
| 9.999 | H60510   | 5.33  | W46682   | 3.645 | W52472   | 3.742 | R84764   |
| 9.915 | R13129   | 5.39  | AA176700 | 6.109 | H09049   | 4.049 | R21449   |
| 9.999 | AA040826 | 3.612 | H47207   | 3.843 | H81775   | 5.074 | AA160325 |
| 2.186 | N76562   | 7.666 | W87527   | 5.664 | H71710   | 2.356 | R97269   |
| 8.468 | R13125   | 5.965 | W47502   | 2.494 | W17237   | 3.68  | H86214   |
| 9.999 | W52400   | 6.338 | N44758   | 3.516 | H06318   | 4.654 | N40829   |
| 3.376 | AA054303 | 2.313 | AA126937 | 4.006 | T63499   | 5.114 | W19928   |
| 4.263 | AA042800 | 6.232 | R31353   | 5.829 | R22383   | 5.526 | H18298   |
| 2.681 | W46165   | 4.846 | R18798   | 3.457 | T87920   | 2.867 | N39173   |
| 8.287 | W24084   | 4.648 | T78246   | 5.766 | W47525   | 3.251 | R76943   |
| 9.999 | T77247   | 6.726 | H29713   | 5.493 | W32575   | 4.139 | W07720   |
| 8.506 | R13073   | 4.524 | AA026304 | 5.622 | R84635   | 3.604 | AA085920 |
| 9.999 | T95699   | 6.113 | AA053162 | 5.284 | R18138   | 3.814 | H18258   |
| 8.7   | N30952   | 1.702 | H65775   | 3.414 | R34648   | 3.192 | AA035131 |
| 8.372 | N29065   | 3.685 | W32710   | 4.46  | H80175   | 2.275 | AA033736 |
| 7.37  | H13744   | 6.264 | T51305   | 4.479 | R76163   | 3.896 | R71065   |
| 9.999 | H46657   | 7.182 | R14403   | 4.381 | W17182   | 3.534 | AA074177 |
| 6.115 | H83517   | 3.9   | W38235   | 5.81  | AA126956 | 4.358 | R92264   |
| 6.587 | AA129780 | 5     | W47083   | 3.781 | AA088390 | 3.561 | N34634   |
| 6.587 | N42542   | 5.209 | W35243   | 4.048 | R52046   | 3.695 | AA114861 |
| 9.999 | H67546   | 3.288 | W24343   | 5.474 | N98916   | 4.673 | R31970   |
| 6.678 | AA181350 | 5.209 | H93373   | 3.226 | R51929   | 3.989 | H70974   |
| 7.187 | T67190   | 6.255 | R26331   | 3.033 | H69334   | 4.646 | AA156193 |
| 6.498 | AA034932 | 2.549 | R70082   | 5.458 | W20511   | 3.258 | AA085385 |
| 9.999 | H41372   | 4.698 | W32969   | 3.742 | T40473   | 4.453 | W49770   |
| 9.999 | H08885   | 4.038 | R66920   | 5.132 | R82723   | 4.056 | W37672   |
| 8.221 | H90627   | 6.586 | W25455   | 5.886 | T50788   | 2.503 | W38097   |
| 4.812 | T91423   | 7.516 | W38478   | 3.266 | AA036758 | 5.118 | N48838   |



|       |          |       |          |       |          |       |          |
|-------|----------|-------|----------|-------|----------|-------|----------|
| 5.535 | N49031   | 1.961 | AA039447 | 3.564 | W46219   | 5.105 | H41937   |
| 7.673 | AA079020 | 3.163 | H89835   | 3.535 | N64406   | 1.544 | AA159807 |
| 5.718 | H44892   | 3.311 | AA156148 | 4.839 | W17076   | 3.436 | AA112478 |
| 2.058 | H38180   | 5.592 | W38424   | 5.314 | H18129   | 4.396 | R07212   |
| 3.101 | T48613   | 6.954 | H75477   | 4.118 | H93835   | 4.917 | AA128281 |
| 3.236 | H12952   | 3.507 | W31707   | 5.306 | AA167017 | 4.303 | N39630   |
| 2.553 | T87585   | 3.968 | N40606   | 5.012 | W24939   | 4.165 | W39234   |
| 2.961 | R76842   | 3.417 | W67757   | 3.887 | W38826   | 1.983 | H68587   |
| 4.505 | N54105   | 5.57  | AA074760 | 3.791 | H62991   | 3.358 | H78277   |
| 3.424 | W72986   | 2.311 | H18350   | 4.853 | AA132801 | 2.968 | T87597   |
| 3.738 | H53662   | 2.707 | W31757   | 2.065 | R54128   | 3.142 | H64978   |
| 1.682 | W05551   | 3.252 | T89571   | 3.35  | AA214334 | 4.376 | W16557   |
| 2.942 | H62026   | 3.202 | W07148   | 3.553 | W16484   | 3.316 | H45068   |
| 3.16  | AA146611 | 2.404 | H66389   | 3.902 | AA122157 | 3.559 | H08997   |
| 3.702 | H70850   | 3.621 | R68331   | 3.451 | H44677   | 3.277 | H08983   |
| 4.118 | W49687   | 4.089 | AA099075 | 3.277 | W20192   | 2.455 | H66256   |
| 4.359 | AA100113 | 4.46  | H91361   | 3.534 | T47067   | 2.632 | T70457   |
| 3.338 | AA133312 | 2.057 | N42428   | 3.581 | T82048   | 2.569 | H13942   |
| 4.367 | T49117   | 3.423 | W48763   | 3.694 | AA134135 | 2.59  | T77584   |
| 3.79  | H58461   | 3.844 | R60387   | 2.79  | R25979   | 2.346 | R71543   |
| 4.806 | N69323   | 3.421 | W19744   | 1.586 | R21064   | 3.168 | T98705   |
| 2.664 | W05089   | 2.473 | R59435   | 1.972 | H29698   | 3.43  | T78542   |
| 3.176 | AA070823 | 3.189 | R07238   | 3.507 | R89708   | 2.887 | T74951   |
| 1.966 | W24455   | 2.496 | AA074340 | 3.189 | R06568   | 3.768 | W16974   |
| 3.258 | R63252   | 3.287 | N78038   | 1.957 | AA009869 | 3.48  | R88098   |
| 4.543 | H80571   | 3.935 | W07144   | 1.627 | W31940   | 2.021 | W48791   |
| 3.593 | AA131550 | 4.133 | R06175   | 3.545 | W38638   | 3.686 | R80458   |
| 4.34  | N42413   | 3.912 | N46036   | 1.634 | R60752   | 1.812 | H29706   |
| 2.313 | R62688   | 2.535 | W78057   | 2.917 | R28459   | 3.501 | AA130339 |

|       |          |       |          |       |          |       |          |
|-------|----------|-------|----------|-------|----------|-------|----------|
| 2.952 | H73881   | 3.614 | R16609   | 3.614 | H63610   | 2.642 | H10811   |
| 5.245 | AA007484 | 3.208 | H01260   | 3.568 | R66182   | 2.323 | AA074067 |
| 6.009 | N57562   | 1.818 | W04913   | 2.77  | H95908   | 2.419 | T56791   |
| 3.62  | H25971   | 3.591 | R61631   | 2.83  | H24644   | 2.432 | R48720   |
| 3.583 | H19106   | 3.528 | H71668   | 2.458 | AA044130 | 2.881 | R20222   |
| 3.085 | W46660   | 4.66  | R18905   | 2.039 | R23341   | 3.274 | H03764   |
| 3.694 | R36401   | 2.311 | H64449   | 3.785 | H01679   | 2.71  | R55247   |
| 3.945 | R09905   | 1.619 | W07452   | 2.82  | T91461   | 3.324 | AA005419 |
| 4.416 | H40081   | 3.462 | AA203284 | 3.202 | T74959   | 2.494 | T97640   |
| 4.498 | AA156956 | 4.843 | N66473   | 1.924 | T39976   | 4.157 | R23556   |
| 3.22  | AA112421 | 3.777 | H78279   | 3.135 | H68817   | 3.956 | N53743   |
| 2.991 | AA147722 | 3.256 | AA156298 | 3.739 | H83559   | 1.947 | W01963   |
| 4.341 | W20171   | 3.25  | N75101   | 1.841 | T79362   | 3.858 | N94762   |
| 1.778 | W21312   | 3.961 | H75277   | 2.551 | N36269   | 3.655 | AA007521 |
| 3.42  | N28517   | 4.366 | R80450   | 3.285 | T79536   | 1.506 | R00903   |
| 3.436 | W07026   | 3.532 | AA057729 | 3.193 | T87507   | 2.775 | H83982   |
| 4.393 | R21898   | 2.373 | T92805   | 3.87  | T71354   | 2.61  | H12686   |
| 3.543 | R36586   | 3.167 | AA005286 | 3.572 | W19860   | 2.577 | H78353   |
| 2.784 | T48691   | 3.33  | H12796   | 2.896 | AA039258 | 3.227 | N45476   |
| 4.06  | W16946   | 1.679 | R60831   | 4.245 | T79534   | 2.799 | R55692   |
| 3.071 | T85481   | 3.64  | T85390   | 2.096 | R59467   | 3.196 | W31182   |
| 4.116 | T83199   | 2.606 | N73091   | 3.168 | H91401   | 2.371 | T91436   |
| 2.479 | H19169   | 2.648 | T56084   | 2.074 | W24718   | 2.951 | T54610   |
| 3.768 | W37172   | 3.4   | W37084   | 2.318 | T54602   | 2.735 | AA001324 |
| 2.146 | H70732   | 3.531 | W07043   | 2.636 | H65057   | 4.007 | H02412   |
| 4.475 | N75228   | 2.86  | N44537   | 3.254 | R52482   | 3.547 | N92284   |
| 3.428 | W00630   | 3.546 | T74332   | 2.377 | H95979   | 2.257 | H78485   |
| 2.951 | N57398   | 2.2   | R35560   | 2.4   | R80523   | 3.216 | R20594   |
| 3.62  | W15521   | 3.945 | R69162   | 2.953 | H79197   | 3.416 | H12419   |

|       |          |       |          |       |          |       |        |
|-------|----------|-------|----------|-------|----------|-------|--------|
| 3.262 | R61036   | 2.337 | T48694   | 3.711 | R85335   | 1.522 | R91771 |
| 2.572 | T68568   | 3.228 | N31889   | 2.546 | T56622   | 3.225 | H09280 |
| 1.5   | N40660   | 2.72  | R60420   | 3.107 | AA205009 | 2.957 | H63806 |
| 2.193 | N98743   | 3.234 | AA004897 | 2.83  | W00950   | 2.588 | R70441 |
| 3.464 | W16685   | 2.893 | R24648   | 2.271 | N73209   | 3.093 | R19326 |
| 2.372 | T82120   | 2.187 | AA063234 | 2.596 | N31231   |       |        |
| 2.765 | T61346   | 3.766 | R96692   | 2.953 | T85879   |       |        |
| 4.054 | AA214079 | 3.217 | AA004891 | 2.655 | R32750   |       |        |
| 1.542 | AA164677 | 3.915 | W00391   | 1.922 | N32733   |       |        |
| 2.937 | AA211776 | 3.291 | R82770   | 3.495 | W17002   |       |        |
| 3.067 | T87472   | 4.545 | H18766   | 2.984 | W20484   |       |        |
| 3.058 | H61812   | 3.167 | R87818   | 1.377 | AA136789 |       |        |
| 2.937 | N45602   | 2.214 | W88806   | 2.364 | H45241   |       |        |
| 2.927 | R14907   | 3.318 | T54086   | 1.678 | W17311   |       |        |
| 3.476 | H12508   | 2.992 | H61280   | 3.688 | R87413   |       |        |
| 3.25  | W19104   | 3.13  | AA167039 | 3.432 | R14301   |       |        |
| 2.441 | R87193   | 3.287 | T87358   | 2.927 | R80475   |       |        |
| 2.521 | H92713   | 2.711 | W03009   | 3.258 | T79546   |       |        |
| 2.617 | R32216   | 2.674 | N98348   | 3.18  | T78497   |       |        |
| 2.262 | AA126184 | 3.311 | N47460   | 1.595 | R52015   |       |        |
| 3.281 | H09869   | 2.187 | H73438   |       |          |       |        |
| 2.02  | AA054041 | 2.751 | W78830   |       |          |       |        |
| 2.639 | H66558   | 2.953 | T49575   |       |          |       |        |
| 3.709 | R07731   | 3.122 | N47660   |       |          |       |        |
| 3.032 | T80874   | 3.435 | H03226   |       |          |       |        |
| 3.673 | H51373   | 3.371 | H42536   |       |          |       |        |
| 3.114 | T51726   | 3.049 | T85153   |       |          |       |        |
| 3.137 | H09884   | 2.421 | N53255   |       |          |       |        |
| 3.202 | T98755   | 2.379 | R70814   |       |          |       |        |

|       |          |       |        |  |  |
|-------|----------|-------|--------|--|--|
| 3.673 | H51373   | 3.371 | H42536 |  |  |
| 3.114 | T51726   | 3.049 | T85153 |  |  |
| 3.137 | H09884   | 2.421 | N53255 |  |  |
| 3.202 | T98755   | 2.379 | R70814 |  |  |
| 3.201 | AA194172 | 2.33  | W47650 |  |  |
| 1.981 | H13009   | 3.072 | T78498 |  |  |

**Table 4. Genes (identified by Genbank Accession Number) whose expression was repressed in hypoxic cells, shown with the ratio of their expression in oxic cells over their expression in hypoxic cells.**

| ratio | Acc. No. | ratio | Acc. No. | ratio | Acc. No. | ratio | Acc. No. |
|-------|----------|-------|----------|-------|----------|-------|----------|
| 9.999 | H38055   | 7.873 | N33752   | 4.55  | R59009   | 9.999 | R31317   |
| 6.948 | AA057425 | 6.351 | T54424   | 3.78  | AA121166 | 6.082 | AA057398 |
| 9.999 | AA116099 | 8.097 | T87470   | 8.936 | R01823   | 6.504 | R89643   |
| 9.999 | AA057428 | 7.137 | H06343   | 7.628 | AA085375 | 7.172 | H70359   |
| 9.999 | R73197   | 4.887 | AA058878 | 3.185 | AA054115 | 5.273 | N48132   |
| 9.999 | R48415   | 9.999 | H46055   | 8.052 | H96006   | 4.543 | AA054096 |
| 9.999 | H14999   | 6.651 | T40066   | 5.324 | T67226   | 5.207 | AA054071 |
| 9.999 | T96535   | 9.672 | R89521   | 5.895 | R62231   | 4.911 | AA078915 |
| 9.999 | H27140   | 9.999 | AA065190 | 4.767 | AA112466 | 4.439 | H52742   |
| 9.999 | T99054   | 7.455 | H43837   | 4.271 | N34169   | 3.092 | T67415   |
| 9.999 | H46382   | 4.601 | W81199   | 5.635 | H51983   | 3.457 | R49895   |
| 9.999 | R90757   | 9.999 | H27344   | 6.176 | N52679   | 4.953 | H16042   |
| 9.999 | AA121402 | 9.999 | H49310   | 6.465 | H50403   | 6.039 | H45590   |
| 9.999 | H38676   | 9.999 | R69813   | 4.812 | H26200   | 6.767 | AA029349 |
| 7.52  | AA057511 | 9.999 | N32666   | 6.495 | N30528   | 6.456 | H51981   |
| 9.999 | N43944   | 2.046 | AA053873 | 7.015 | H47146   | 5.186 | R61165   |
| 9.999 | AA134018 | 9.999 | AA125970 | 5.891 | R11999   | 2.541 | R85183   |

|       |          |       |          |       |          |       |          |
|-------|----------|-------|----------|-------|----------|-------|----------|
| 5.741 | H14566   | 4.712 | H83338   | 5.442 | N24303   | 4.764 | H85692   |
| 9.999 | AA035019 | 9.999 | R94457   | 3.989 | H14332   | 5.288 | DROS-A8  |
| 9.285 | N29018   | 4.545 | N31674   | 6.206 | R64420   | 4.603 | R24904   |
| 7.813 | AA069149 | 5.6   | T50828   | 6.203 | R37898   | 3.307 | W72875   |
| 9.999 | R76214   | 7.861 | R74161   | 6.251 | R76298   | 4.033 | H71729   |
| 9.999 | R23999   | 7.437 | T99984   | 5.33  | N98261   | 6.431 | H14193   |
| 9.999 | R23880   | 5.5   | R48041   | 6.206 | R24405   | 8.731 | N48042   |
| 9.999 | AA078826 | 7.565 | H82390   | 4.865 | H39089   | 4.063 | H70778   |
| 7.739 | T92655   | 4.998 | T54422   | 9.999 | W72342   | 6.486 | W24476   |
| 6.577 | W01565   | 6.317 | H98046   | 4.976 | T65484   | 9.999 | W01642   |
| 9.59  | R90884   | 7.321 | N30514   | 3.745 | H96724   | 7.103 | T98068   |
| 5.535 | R56663   | 5.137 | H38881   | 9.999 | H49809   | 3.784 | AA100388 |
| 8.157 | W00931   | 8.737 | T99046   | 3.286 | N57334   | 4.15  | H60927   |
| 9.999 | H50385   | 5.465 | H47440   | 8.214 | R51931   | 3.897 | R28248   |
| 8.922 | H28503   | 6.734 | N42979   | 3.912 | T79680   | 7.319 | H56754   |
| 5.182 | H84008   | 6.802 | W01319   | 3.824 | H21568   | 4.617 | R92111   |
| 7.947 | W01051   | 7.572 | R95136   | 5.746 | T80382   | 6.422 | H94177   |
| 9.05  | R66879   | 9.999 | AA112231 | 9.999 | R87923   | 4.375 | R87352   |
| 9.999 | H45773   | 6.035 | H53489   | 5.902 | R23778   | 6.6   | R31364   |
| 9.999 | AA126109 | 5.53  | N94798   | 8.584 | AA101044 | 3.713 | AA059302 |
| 9.999 | R54784   | 6.009 | N30964   | 3.638 | AA112340 | 5.179 | H51160   |
| 9.999 | N98857   | 9.999 | N44142   | 8.09  | R90895   | 5.423 | R25798   |
| 9.999 | AA056159 | 7.759 | W03129   | 6.3   | N90458   | 4.448 | R63455   |
| 9.999 | R77028   | 4.156 | W03125   | 4.874 | R87886   | 6.596 | T77139   |
| 9.999 | N36070   | 7.184 | R79618   | 4.621 | N32679   | 4.909 | R63498   |
| 5.356 | R89245   | 4.281 | H38147   | 7.755 | W02372   | 4.523 | R22272   |
| 9.999 | H51782   | 4.445 | R38004   | 4.624 | H30637   | 6.954 | H45355   |
| 9.999 | N48735   | 9.999 | R88190   | 8.387 | R01888   | 5.624 | R75964   |

|       |          |       |          |       |          |       |          |
|-------|----------|-------|----------|-------|----------|-------|----------|
| 5.379 | H86672   | 9.999 | N41573   | 8.644 | AA070426 | 4.742 | N32681   |
| 9.999 | T95210   | 9.723 | H52741   | 9.999 | W31524   | 4.494 | R06552   |
| 7.355 | R81942   | 4.401 | T54426   | 6.574 | H81786   | 9.097 | R87320   |
| 9.999 | N53883   | 4.914 | AA054102 | 3.719 | N40437   | 5.369 | R55451   |
| 7.685 | N24364   | 6.874 | R31243   | 7.48  | N42402   | 7.075 | R84765   |
| 7.473 | H91761   | 7.618 | AA113044 | 6.86  | R32757   | 4.058 | H84844   |
| 4.537 | W16945   | 5.394 | R96571   | 8.926 | H21214   | 6.525 | W21173   |
| 4.091 | DROS-A8  | 2.424 | R71723   | 4.111 | H18154   | 1.955 | AA054211 |
| 9.999 | AA115819 | 4.366 | H86277   | 2.981 | R53678   | 3.214 | N50075   |
| 4.996 | N36347   | 2.214 | T53945   | 3.552 | R06539   | 3.359 | T93912   |
| 5.587 | N30932   | 5.37  | R09668   | 4.474 | H43816   | 4.156 | T77415   |
| 2.24  | R80470   | 4.809 | H40716   | 6.663 | AA146629 | 2.779 | AA040227 |
| 2.746 | H16193   | 2.192 | R70132   | 4.402 | R01530   | 3.468 | N26148   |
| 6.109 | AA004785 | 2.555 | R81899   | 1.884 | H86896   | 2.744 | R26215   |
| 3.155 | N28396   | 4.968 | N42806   | 4.013 | R68198   | 3.757 | H58331   |
| 3.525 | AA047581 | 2.243 | H66535   | 3.632 | H16160   | 2.892 | N34217   |
| 4.396 | H84204   | 4.049 | R46282   | 1.867 | R85333   | 2.987 | AA059324 |
| 6.795 | R54918   | 5.522 | R49786   | 1.993 | W16980   | 3.314 | R23635   |
| 5.972 | R78728   | 8.225 | T67978   | 2.042 | T60294   | 1.29  | R77994   |
| 4.684 | R80601   | 3.362 | R81838   | 3.009 | W32352   | 3.188 | R84692   |
| 7.734 | R73050   | 5.364 | H19572   | 1.589 | W15194   | 3.837 | N75231   |
| 4.832 | H12682   | 4.739 | R33409   | 2.503 | H52012   | 4.043 | AA043598 |
| 6.87  | N56601   | 3.959 | AA069498 | 2.192 | R22397   | 2.645 | R22392   |
| 4.153 | R80286   | 4.115 | H44733   | 4.219 | R55158   | 3.933 | R11541   |
| 9.999 | N73428   | 5.33  | AA029010 | 3.069 | AA100975 | 3.244 | H73503   |
| 2.754 | AA002135 | 9.999 | AA113853 | 8.61  | AA113299 | 2.909 | AA053288 |
| 2.948 | H39058   | 4.264 | N31362   | 1.999 | R87373   | 3.059 | H38003   |
| 6.235 | R01799   | 2.793 | R20924   | 4.285 | N45686   | 4.04  | AA088387 |

|       |          |       |          |       |          |       |          |
|-------|----------|-------|----------|-------|----------|-------|----------|
| 6.179 | R09942   | 3.664 | R98517   | 3.87  | W02494   | 4.006 | H89896   |
| 1.849 | R72766   | 4.656 | N24477   | 3.84  | R73063   | 4.744 | AA126881 |
| 2.963 | R82691   | 4.443 | AA032034 | 2.614 | H14441   | 6.651 | AA088231 |
| 2.716 | R83247   | 3.799 | AA054203 | 4.806 | AA058898 | 3.346 | N59684   |
| 9.375 | AA070943 | 2.852 | W95433   | 1.683 | H89823   | 4.122 | T92415   |
| 4.051 | N43796   | 5.221 | N72527   | 4.392 | AA037418 | 3.81  | AA088190 |
| 4.754 | N46182   | 5.408 | W02353   | 4.142 | T83106   | 2.846 | N32669   |
| 5.892 | R32571   | 4.626 | W01717   | 4.241 | T81175   | 4.096 | T58881   |
| 3.03  | W72046   | 7.031 | W79028   | 3.335 | R31471   | 2.097 | T99924   |
| 5.486 | H85829   | 4.702 | R12648   | 3.253 | R86304   | 3.441 | AA046822 |
| 5.718 | R75796   | 3.427 | N32672   | 2.914 | H86156   | 3.813 | H85193   |
| 2.697 | N45640   | 3.989 | N90836   | 4.004 | H87311   | 3.134 | R91315   |
| 2.888 | H49806   | 3.692 | N92684   | 2.595 | T94530   | 3.132 | T71293   |
| 5.419 | H52350   | 2.552 | W06829   | 2.108 | H27617   | 3.45  | H79957   |
| 4.515 | AA071099 | 2.537 | H53375   | 1.565 | H14143   | 3.198 | AA058615 |
| 3.535 | H51993   | 4.752 | H67462   | 4.018 | H49818   | 3.309 | N34153   |
| 3.287 | AA069031 | 4.076 | AA029883 | 1.677 | R97857   | 3.734 | H61493   |
| 5.108 | R79519   | 4.394 | H12075   | 3.544 | N90841   | 1.687 | R56760   |
| 5.336 | N29042   | 3.253 | T86612   | 3.901 | AA076660 | 1.796 | AA132756 |
| 4.075 | R48060   | 9.23  | T85558   | 4.49  | N53295   | 2.934 | H20613   |
| 4.343 | N77703   | 5.32  | N29155   | 1.67  | AA190622 | 6.358 | AA129486 |
| 5.491 | W56898   | 3.579 | H44664   | 4.118 | AA099647 | 3.07  | H44888   |
| 2.202 | H28534   | 5.57  | W16814   | 4.007 | AA069114 | 3.583 | W04937   |
| 4.449 | H62445   | 2.133 | R85191   | 2.606 | W92014   | 2.521 | AA047388 |
| 4.196 | R90798   | 2.161 | H50471   | 4.047 | W87655   | 4.349 | R52735   |
| 7.226 | R35606   | 3.275 | H30629   | 3.891 | W01911   | 2.165 | AA065193 |
| 3.224 | R25899   | 4.284 | R80273   | 4.32  | R54194   | 4.009 | N42453   |
| 9.999 | AA113119 | 4.063 | H27411   | 2.085 | N57249   | 3.688 | W72149   |

|       |          |       |          |       |          |       |          |
|-------|----------|-------|----------|-------|----------|-------|----------|
| 4.2   | N30471   | 4.231 | H44693   | 3.472 | R74281   | 4.603 | AA044942 |
| 5.466 | T98994   | 2.471 | W47021   | 4.044 | AA054209 | 3.445 | AA187560 |
| 2.577 | AA075652 | 4.544 | H69415   | 2.621 | H62639   | 2.273 | R74076   |
| 2.448 | R06926   | 2.425 | AA181061 | 1.461 | T78546   | 2.268 | H65149   |
| 2.535 | W45582   | 3.336 | T97872   | 3.677 | W40228   | 5.781 | T48877   |
| 4.173 | T82054   | 3.553 | H51540   | 6.08  | AA101477 | 3.152 | AA033945 |
| 3.359 | T98110   | 2.63  | N44493   | 3.907 | W00916   | 3.032 | H49111   |
| 2.703 | R86735   | 2.587 | AA088784 | 1.846 | N90969   | 3.741 | H22503   |
| 2.77  | AA045397 | 3.082 | R25304   | 3.006 | H83363   | 2.252 | N78086   |
| 2.893 | R53671   | 3.06  | AA058600 | 4.484 | W17108   | 2.755 | H71635   |
| 2.077 | AA088407 | 2.741 | H81782   | 1.482 | N64281   | 2.673 | N77126   |
| 2.213 | T95166   | 3.466 | H84604   | 2.899 | AA045672 | 4.796 | AA083226 |
| 1.963 | H27400   | 7.264 | AA113169 | 2.994 | H43746   |       |          |
| 5.333 | T62191   | 3.859 | N63192   | 2.65  | R91004   |       |          |
| 2.671 | N32657   | 2.887 | N29162   | 2.439 | AA047858 |       |          |
| 3.374 | T83919   | 1.926 | R28329   | 2.867 | W21593   |       |          |
| 3.108 | AA191189 | 3.17  | AA172313 | 2.374 | N34202   |       |          |
| 2.292 | R74157   | 2.057 | R34929   | 3.144 | N80131   |       |          |
| 1.787 | R21095   | 4.695 | W55902   | 2.458 | AA043774 |       |          |
| 4.011 | AA180772 | 2.179 | R13273   | 3.306 | T56558   |       |          |
| 2.901 | H82540   | 2.354 | AA121148 | 3.896 | AA205980 |       |          |
| 3.707 | R73398   | 5.805 | AA088299 | 2.565 | AA179841 |       |          |
| 2.658 | H55982   | 2.233 | H84617   | 2.973 | H68783   |       |          |
| 1.826 | H45128   | 2.011 | AA059207 | 3.142 | W30866   |       |          |
| 4.006 | W25183   | 2.742 | H39778   | 2.992 | AA028961 |       |          |
| 4.299 | T56400   | 3.034 | H83022   | 2.489 | R26026   |       |          |
| 4.086 | T95526   | 3.245 | AA113900 | 5.751 | AA085171 |       |          |
| 1.691 | AA053901 | 2.716 | R19726   | 1.822 | AA150817 |       |          |



Example 9. Analysis of Gene Expression under Hypoxia using GEM<sup>TM</sup> microarrays

5           The hypoxic induction of genes in FaDu cells was analyzed by comparing the expression of genes in FaDu cells exposed to hypoxic conditions (5% CO<sub>2</sub>/5% H<sub>2</sub>/90% N<sub>2</sub> for 16 hours at 37°C) to those exposed to normal, oxic conditions. This differential expression was analyzed using GEM<sup>TM</sup> technology provided by Genome Systems Inc. Messenger RNA (mRNA) was extracted from hypoxic  
10 FaDu cells, and separately from oxic FaDu cells.

          The total RNA was isolated from the cells essentially according to the standard Genome Systems Inc. protocol, as follows. 500 µl Trizol was added 50-100 mg of fresh frozen cells. The cells were then immediately homogenized. 500 µl Trizol was then added, and the sample was mixed well. The sample was  
15 homogenized for five minutes at room temperature. Next, 0.2 ml chloroform was added per 1 ml Trizol. The mixture was shaken vigorously for 15 seconds and then allowed to incubate three minutes at room temperature. The sample was then centrifuged at 12,000X g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh centrifuge tube without disturbing the interphase. 0.5 ml of  
20 isopropanol was added and the samples were incubated for 10 minutes at room temperature. The RNA was pelleted by centrifuging at 12,000X g for 10 minutes at 4°C. The supernatant was then removed. 1 ml of 75% ethanol was added to the pellet, which was then vortexed. This was followed by centrifugation at 7,500X g for 5 minutes at 4°C. The ethanol was removed. The pellet was dried for 10  
25 minutes at room temperature and then dissolved in 10 µl nuclease-free water and stored at -80°C.

          Next, the poly A<sup>+</sup> RNA was isolated from total RNA essentially according to the standard Genome Systems Inc. protocol, as follows. To purify polyA RNA,

the total RNA sample was passed twice over OligoTex mRNA isolation columns from Qiagen. After the elution of the polyA RNA, the polyA RNA was ethanol precipitated, and the final product was brought up in DEPC H<sub>2</sub>O or TE. For 50 µl of elution from the OligoTex column, 40 µl of 1X TE and 1 µl of glycogen (5 mg/ml) was added. Then 120 µl of 100% EtOH was added and the sample was frozen at -80°C for 10 minutes. The sample was then spun at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and 250 µl of 75% EtOH was added. The pellet was spun at 12,000 x g for 5 minutes at 4°C. The supernatant was again removed and the pellet dried for 10 minutes at room temperature. The pellet was then dissolved in DEPC H<sub>2</sub>O to a concentration of 50 ng/µl.

The purified RNA samples were sent to Genome Systems Inc. to perform a GEM microarray analysis. From the mRNA samples, fluorescent labeled cDNA probes were prepared by Genome Systems Inc. using standard methodologies familiar to those skilled in the art. The cDNA probes corresponding to the mRNA sample from the oxic FaDu cells were labeled with a different, distinguishable fluorescent label than the cDNA probes corresponding to the mRNA sample from the hypoxic FaDu cells.

The two fluorescent probe samples (one from hypoxic FaDu cells, the other from oxic FaDu cells) were then simultaneously applied by Genome Systems Inc. to their Human UniGEM V microarray for hybridization to the arrayed cDNA molecules. The Human UniGEM V microarray contains sequence verified Genome Systems Inc. proprietary cDNA clones representing more than 4,000 known human genes and up to 3,000 ESTs mapped to the UniGene database. (All of the genes on the microarray were selected for criteria such as known functions, homologies, and presence on the human transcript map.) The genes or gene fragments of the GEM microrarray (each 500-5000 base pairs in length) are arrayed on glass surface to which they have been chemically bonded.

Once the two fluorescent cDNA samples were sufficiently incubated with the arrayed cDNA molecules to allow for hybridization to occur, the microarray was washed free of probe molecules which had not hybridized. The different gene/EST sites of the GEM microarray are then scanned for the each of the two fluorescent labels. Presence of the fluorescent label at a particular gene site indicates the expression of that gene in the cell corresponding to that fluorescent label.

The 30 genes or ESTs which were determined on the microarray to have the greatest level of induction in hypoxic cells (versus oxic cells) are listed below in Table 5, along with their levels of induction, functional category if known, and GenBank accession number.

**Table 5. Genes Induced by Hypoxia in FaDu cells.**

| <b>Functional Category</b> | <b>Gene</b>                                  | <b>GenBank #</b> | <b>Induction</b> |
|----------------------------|--|------------------|------------------|
| <b>Angiogenesis</b>        | fibroblast growth factor (FGF-3)             | X14445           | 5.6              |
|                            | vascular endothelial growth factor (VEGF)    | X54936           | 4.0              |
|                            | EPH receptor ligand                          | M57730           | 3.9              |
| <b>Tissue Remodeling</b>   | plasminogen activator inhibitor -1 (PAI-1)   | M14083           | 13.5             |
|                            | macrophage migration inhibitory factor (MIF) | M25639           | 9.3              |
|                            | fibronectin receptor                         | X06256           | 6.0              |
|                            | lysyl hydroxylase-2                          | U84573           | 4.8              |
|                            | endothelin-2                                 | M65199           | 4.4              |
|                            | B-cell translocation gene-1 (BTG-1)          | X61123           | 4.9              |

|                      |  |         |      |
|----------------------|--|---------|------|
| <b>Cell Cycle</b>    | reducing agent and tunicamycin-responsive protein (RTP)            | D87953  | 3.8  |
|                      | CDC-like kinase (clk-1)  | L29219  | 3.0  |
|                      | quiescin (Q6)  | U97276  | 2.9  |
|                      | growth arrest DNA damage-inducible protein 45 (GADD45)             | L24498  | 2.9  |
| <b>Miscellaneous</b> | Differentiation of Embryo  | AB0040  | 8.2  |
|                      | Chondrocytes-1 (DEC1)  | 66      |      |
|                      | low density lipoprotein receptor (LDLR)                            | X13916  | 6.0  |
|                      | related protein  |         |      |
|                      | human hairy gene homologue (HRY)                                   | L19314  | 4.7  |
|                      | adipophilin  | X97324  | 3.9  |
| <b>Metabolism</b>    | cyclooxygenase-1 (COX-1)   | U63846  | 3.0  |
|                      | fructose bisphosphatase  | AF0549  | 4.5  |
|                      |  | 87      |      |
|                      | creatine transporter   | U36341, | 4.1  |
|                      |  | U41163  |      |
|                      | fatty acid binding protein   | M94856  | 3.9  |
|                      | glucose transporter-like protein III (GLUT-3)                      | M20681  | 3.4  |
| <b>Apoptosis</b>     | lactate dehydrogenase (LDH)  | X02152  | 2.9  |
|                      | insulin-like growth factor (IGFBP-3)                               | M35878  | 11.1 |
|                      | Bcl-2-interacting killer (BIK)                                     | X89986  | 7.6  |
|                      | 19 kDa-interacting protein 3 long/Nip3-like protein X (NipP3L/Nix) | AB0047  | 5.3  |
|                      | Pim-1  | 88      |      |
|                      |  | M54915  | 4.4  |
|                      | EST  | AA0447  | 5.0  |
|                      |  | 68      |      |

|                |          |        |     |
|----------------|----------|--------|-----|
|                | 71       |        |     |
|                | EST      | AA0545 | 4.7 |
|                |          | 43     |     |
|                | KIAA0242 | D87684 | 3.9 |
| <b>Unknown</b> | EST      | AA1562 | 3.5 |
|                |          | 40     |     |
|                | EST      | AA1868 | 3.4 |
|                |          | 03     |     |
|                | EST      | AA0630 | 3.0 |
|                |          | 84     |     |
|                | EST      | H15429 | 2.9 |
|                | EST      | U60873 | 2.9 |

Various modifications and variations of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with

5 specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art are intended to be within the scope of the claims.

What is claimed is:

1. An isolated polynucleotide comprising:  
the sequence set forth in SEQ ID NO:1, the complement thereof, an  
5 at least twelve nucleotide-long fragment thereof, or a sequence which hybridizes  
thereto; or  
the sequence set forth in SEQ ID NO:3, the complement thereof, an  
at least twelve nucleotide-long fragment thereof, or a sequence which hybridizes  
thereto.  
10
2. The isolated polynucleotide of Claim 1, which comprises nucleotides 62-  
343 of SEQ ID NO:1, the complement thereof, an at least twelve nucleotide-long  
fragment thereof, or a sequence which hybridizes thereto.
- 15 3. The isolated polynucleotide of Claim 1, which comprises nucleotides 274-  
465 of SEQ ID NO:3, the complement thereof, an at least twelve nucleotide-long  
fragment thereof, or a sequence which hybridizes thereto.
4. An expression vector comprising:  
20 (i) the polynucleotide of Claim 1; and  
(ii) a promoter, wherein said promoter is operably linked to said  
polynucleotide.
5. A delivery vehicle comprising the polynucleotide of Claim 1.  
25
6. An isolated cell comprising the polynucleotide of Claim 1.

7. An antisense oligonucleotide capable of blocking expression of the polynucleotide of Claim 1.
8. A probe comprising a polynucleotide of Claim 1, or a fragment thereof, that  
5 is at least 12 nucleotides in length.
9. An array of polynucleotides, comprising:
- (a) at least one polynucleotide of Claim 1; and
  - (b) a second polynucleotide, wherein said second polynucleotide  
10 comprises the sequence of a second hypoxia-inducible gene, or an at least twelve nucleotide-long fragment thereof.
10. An isolated polypeptide encoded by the polynucleotide sequence of Claim 1, or a biochemically equivalent fragment thereof.  
15
11. An isolated polypeptide comprising SEQ ID NO:2, a biochemically equivalent fragment of SEQ ID NO:2, SEQ ID NO:4, or a biochemically equivalent fragment of SEQ ID NO:4.
- 20 12. An array of polypeptides, comprising:
- (a) at least one polypeptide of Claim 11; and
  - (b) at least one of a second polypeptide, wherein said second polypeptide is a hypoxia-induced gene product or a biochemically equivalent fragment thereof.  
25
13. An antibody specifically immunoreactive with a polypeptide of Claim 11.

14. An array of antibodies, comprising:

(a) at least one antibody of Claim 13; and

(b) at least one of a second antibody, wherein said second antibody specifically binds a second hypoxia-induced gene product or a fragment thereof.

5

15. An array of polynucleotides, comprising at least two different hypoxia-inducible genes, or complements thereto, or at least twelve nucleotide-long fragments thereof, or sequences which hybridize thereto.

10 16. The array of Claim 15, comprising at least two different polynucleotides, each comprising a hypoxia-inducible gene, or an at least twelve nucleotide-long fragments thereof, or the complement thereto, wherein said hypoxia-inducible genes encode proteins belonging to different functional categories selected from the group consisting of glycolytic enzymes/proteins, metabolic/homeostatic  
15 proteins, apoptosis proteins, DNA repair proteins, angiogenesis/tissue remodeling proteins, cell-cycle proteins, and erythropoiesis/vascular regulatory proteins.

17. The array of Claim 15, comprising at least two different polynucleotides, each comprising a hypoxia-inducible gene, or an at least twelve nucleotide-long  
20 fragment thereof, or the complement thereto, wherein said hypoxia-inducible genes all encode proteins belonging to a single functional category selected from the group consisting of glycolytic enzymes/proteins, metabolic/homeostatic proteins, apoptosis proteins, DNA repair proteins, angiogenesis/tissue remodeling proteins, cell-cycle proteins, and erythropoiesis/vascular regulatory proteins.

25

18. The array of Claim 17, comprising at least two different polynucleotides, each comprising a hypoxia-inducible gene, or an at least twelve nucleotide-long



fragment thereof, or the complement thereto, wherein all of the hypoxia-inducible genes encode angiogenesis or tissue remodeling proteins.

19. The array of Claim 15, comprising:

- 5 (a) at least one gene selected from the group consisting of *HIG1*, *HIG2*, *annexin V*, *lipocortin 2*, *hnRNP A1*, *Ku autoantigen*, *phosphoribosylpyrophosphate synthetase*, *acetoacetylCoA thiolase*, *ribosomal L7*, *fibroblast growth factor-3*, *EPH receptor ligand*, *plasminogen activator inhibitor-1*, *macrophage migration inhibitory factor*, *fibronectin receptor*, *lysyl*  
10 *hydroxylase-2*, *endothelin-2*, *B-cell translocation gene-1*, *reducing agent and tunicamycin-responsive protein*, *CDC-like kinase-1*, *quiescin*, *growth arrest DNA damage-inducible protein 45*, *DEC1*, *low density lipoprotein receptor related protein*, *hamster hairy gene homologue*, *adipophilin*, *cyclooxygenase-1*, *fructose biphosphatase*, *creatine transporter*, *fatty acid binding protein*, *lactate*  
15 *dehydrogenase*, *Bcl-2-interacting killer*, *Nip3L/Nix*, and *Pim-1*, or an at least twelve nucleotide-long fragment thereof; and

(b) a second polynucleotide, wherein said second polynucleotide comprises a second hypoxia-inducible gene or an at least twelve nucleotide-long fragment thereof.

20

20. An array of polypeptides, comprising the polypeptide expression products of at least two hypoxia-inducible genes, or biochemically equivalent fragments thereof.

- 25 21. The array of Claim 20, comprising at least two different hypoxia-induced proteins, or biochemically equivalent fragments thereof, wherein each hypoxia-induced protein belongs to a different functional category selected from the group consisting of glycolytic proteins, metabolic enzymes/proteins, apoptosis proteins,

DNA repair proteins, angiogenesis/tissue remodeling proteins, cell-cycle proteins, and erythropoiesis/vascular regulatory proteins.

22. The array of Claim 20, comprising at least two different hypoxia-induced  
5 proteins or biochemically equivalent fragments thereof, wherein said hypoxia-induced proteins are all proteins belonging to a single functional category selected from the group consisting of glycolytic enzymes/proteins, metabolic/homeostatic proteins, apoptosis proteins, DNA repair proteins, angiogenesis/tissue remodeling proteins, cell-cycle proteins, and erythropoiesis/vascular regulatory proteins.
- 10 23. The array of Claim 20, comprising:
- (a) at least one protein selected from the group consisting of HIG1, HIG2, annexin V, lipocortin 2, hnRNP A1, Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7,  
15 fibroblast growth factor-3, EPH receptor ligand, plasminogen activator inhibitor-1, macrophage migration inhibitory factor, fibronectin receptor, lysyl hydroxylase-2, endothelin-2, B-cell translocation gene-1, reducing agent and tunicamycin-responsive protein, CDC-like kinase-1, quiescin, growth arrest DNA damage-inducible protein 45, DEC1, low density lipoprotein receptor related protein,  
20 hamster hairy gene homologue, adipophilin, cyclooxygenase-1, fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1, or a biochemically equivalent fragment thereof; and
- (b) at least one of a second polypeptide, wherein said second  
25 polypeptide is a second hypoxia-induced gene product, or a biochemically equivalent fragment thereof.

24. An array of antibodies, comprising at least two different antibodies specifically immunoreactive with the polypeptide expression products of hypoxia-inducible genes.

5 25. An array of antibodies of Claim 24, comprising:

(a) at least one antibody immunoreactive with a protein selected from the group consisting of HIG1, HIG2, annexin V, lipocortin 2, hnRNP A1, Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3, EPH receptor ligand, plasminogen  
10 activator inhibitor-1, macrophage migration inhibitory factor, fibronectin receptor, lysyl hydroxylase-2, endothelin-2, B-cell translocation gene-1, reducing agent and tunicamycin-responsive protein, CDC-like kinase-1, quiescin, growth arrest DNA damage-inducible protein 45, DEC1, low density lipoprotein receptor related protein, hamster hairy gene homologue, adipophilin, cyclooxygenase-1, fructose  
15 bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1; and

(b) at least one of a second antibody, wherein said second antibody specifically binds a second hypoxia-induced gene product or a biochemically equivalent fragment thereof.

20

26. A method of assaying for expression of hypoxia-inducible genes in a tissue of an animal, comprising:

(a) contacting the proteins of a sample of body fluid or tissue obtained from said animal with the array of Claim 24; and

25 (b) detecting the amount and position of protein from said sample that binds to the array.

27. A method of evaluating a hypoxia-related condition in a tissue of an animal, comprising:

(a) contacting the proteins of a sample of body fluid or tissue obtained from said animal with the array of Claim 24; and

5 (b) detecting the amount and position of protein from said sample that binds to the array.

28. The method of Claim 27, wherein said hypoxia-related condition is cancer, ischemia, reperfusion, retinopathy, neonatal distress, preeclampsia, cardiac arrest,  
10 or stroke.

29. A method of diagnosing a hypoxia-related condition in an animal, said method comprising:

(a) evaluating the hypoxia-related condition in a tissue of the animal by  
15 the method of Claim 27; and

(b) correlating the result of the determination of step(a) with an appropriate treatment for the animal.

30. A method of treating a hypoxia-related condition in a tissue of an animal,  
20 said method comprising:

(a) diagnosing the hypoxia-related condition in the tissue of the animal by the method of Claim 29; and

(b) treating said animal with said appropriate treatment.

25 31. A method of determining the presence of hypoxia in a tissue in an animal, comprising:

(a) contacting the proteins of a sample of body fluid or tissue obtained from said animal with the array of Claim 24; and

(b) detecting the amount and position of protein from said sample that binds to the array.

32. A method of assaying for expression of hypoxia-inducible genes in a tissue  
5 of an animal, comprising:

(a) contacting messenger RNA from a sample of body fluid or tissue obtained from said animal, or cDNA derived therefrom, with the array of Claim 15; and

(b) detecting the amount and position of messenger RNA or cDNA from  
10 said sample that binds to the array.

33. A method of evaluating a hypoxia-related condition in a tissue of an animal, comprising:

(a) contacting messenger RNA from a sample of body fluid or tissue  
15 obtained from said animal, or cDNA derived therefrom, with the array of Claim 15; and

(b) detecting the amount and position of the messenger RNA or the cDNA that binds to the array.

34. The method of Claim 33, wherein said hypoxia-related condition is cancer,  
20 ischemia, reperfusion, retinopathy, neonatal distress, preeclampsia, cardiac arrest, or stroke.

35. A method of diagnosing a hypoxia-related condition in an animal, said method comprising:

25 (a) evaluating the hypoxia-related condition in a tissue of the animal by the method of Claim 33; and

(b) correlating the result of the determination of step (b) with an appropriate treatment for the animal.

36. A method of treating a hypoxia-related condition in an animal, said method comprising:

(a) diagnosing the hypoxia-related condition in the tissue of the animal by the method of Claim 35; and

5 (c) treating said animal with said appropriate treatment.

37. A method of determining the presence of hypoxia in a tissue in an animal, comprising:

(a) contacting messenger RNA from a sample of body fluid or tissue  
10 obtained from said animal, or cDNA derived therefrom, with the array of Claim 15; and

(b) detecting the amount and position of the messenger RNA or the cDNA that binds to the array.

15 38. A method of treating a hypoxia-related condition in a tissue in an animal, comprising:

(a) assaying for either the mRNA transcript or the polypeptide expression product of at least one gene selected from the group consisting of *HIG1*, *HIG2*, *annexin V*, *lipocortin 2*, *hnRNP A1*, *Ku autoantigen*,  
20 *phosphoribosylpyrophosphate synthetase*, *acetoacetylCoA thiolase*, *ribosomal L7*, *fibroblast growth factor-3*, *EPH receptor ligand*, *plasminogen activator inhibitor-1*, *macrophage migration inhibitory factor*, *fibronectin receptor*, *lysyl hydroxylase-2*, *endothelin-2*, *B-cell translocation gene-1*, *reducing agent and tunicamycin-responsive protein*, *CDC-like kinase-1*, *quiescin*, *growth arrest DNA*  
25 *damage-inducible protein 45*, *DEC1*, *low density lipoprotein receptor related protein*, *hamster hairy gene homologue*, *adipophilin*, *cyclooxygenase-1*, *fructose biphosphatase*, *creatine transporter*, *fatty acid binding protein*, *glucose*

*transporter-like protein III, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1* in a body fluid or the tissue of said animal;

(b) correlating the result of the determination of step (a) with an appropriate treatment for the hypoxia-related condition; and

5 (c) treating said tissue with said appropriate treatment.

39. The method of Claim 38 wherein said hypoxia-related condition is cancer, ischemia, reperfusion, retinopathy, neonatal distress, preeclampsia, cardiac arrest, or stroke.

10

40. The method of Claim 39, wherein

(a) said hypoxia-related condition is cancer; and

(b) said appropriate treatment is selected from the group consisting of radiation therapy, chemotherapy, and surgery.

15 41. A method of determining the presence of hypoxia in a tissue in an animal, comprising:

assaying for either the mRNA transcript or the polypeptide expression product of a gene selected from the group consisting of *HIG1*, *HIG2*, *annexin V*, *lipocortin 2*, *hnRNP A1*, *Ku autoantigen*, *phosphoribosylpyrophosphate*

20 *synthetase*, *acetoacetylCoA thiolase*, *ribosomal L7*, *fibroblast growth factor-3*, *EPH receptor ligand*, *plasminogen activator inhibitor-1*, *macrophage migration inhibitory factor*, *fibronectin receptor*, *lysyl hydroxylase-2*, *endothelin-2*, *B-cell translocation gene-1*, *reducing agent and tunicamycin-responsive protein*, *CDC-like kinase-1*, *quiescin*, *growth arrest DNA damage-inducible protein 45*, *DEC1*,  
25 *low density lipoprotein receptor related protein*, *hamster hairy gene homologue*, *adipophilin*, *cyclooxygenase-1*, *fructose biphosphatase*, *creatine transporter*, *fatty acid binding protein*, *glucose transporter-like protein III*, *lactate*

*dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1* in a body fluid or the tissue of said animal.

42. The method of Claim 41, wherein said tissue is a tumor.

5

43. A method of treating a tumor, comprising:

(a) determining the presence of hypoxia in a tumor by the method of Claim 42; and

(b) treating said tumor with an established form of therapy for cancer.

10

44. The method of Claim 43, wherein said established form of therapy for cancer is selected from the group consisting of radiation therapy, chemotherapy, and surgery.

15 45. The method of diagnosing a hypoxia-related condition in an animal, comprising:

determining the presence of hypoxia in a tissue in the animal by the method of Claim 41.

20 46. A method of attenuating the hypoxic response of tissue in an animal, comprising:

inhibiting the expression of a gene selected from the group consisting of *HIG1*, *HIG2*, *annexin V*, *lipocortin 2*, *hnRNP A1*, *Ku autoantigen*, *phosphoribosylpyrophosphate synthetase*, *acetoacetylCoA thiolase*, *ribosomal L7*,  
25 *fibroblast growth factor-3*, *EPH receptor ligand*, *plasminogen activator inhibitor-1*, *macrophage migration inhibitory factor*, *fibronectin receptor*, *lysyl hydroxylase-2*, *endothelin-2*, *B-cell translocation gene-1*, *reducing agent* and *tunicamycin-responsive protein*, *CDC-like kinase-1*, *quiescin*, *growth arrest DNA*



*damage-inducible protein 45, DEC1, low density lipoprotein receptor related protein, hamster hairy gene homologue, adipophilin, cyclooxygenase-1, fructose biphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1.*

5

47. A method of treating a hypoxia-related condition in an animal, comprising:  
attenuating the hypoxic response of a tissue in said animal by the method of  
Claim 46.

10

48. A method of attenuating the hypoxic response of a tissue, comprising:  
neutralizing a protein selected from the group consisting of *HIG1, HIG2, annexin V, lipocortin 2, hnRNP A1, Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3, EPH receptor ligand, plasminogen activator inhibitor-1, macrophage migration inhibitory factor, fibronectin receptor, lysyl hydroxylase-2, endothelin-2, B-cell translocation gene-1, reducing agent and tunicamycin-responsive protein, CDC-like kinase-1, quiescin, growth arrest DNA damage-inducible protein 45, DEC1, low density lipoprotein receptor related protein, hamster hairy gene homologue, adipophilin, cyclooxygenase-1, fructose biphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1.*

15

20

49. A method of treating a hypoxia-related condition in an animal, comprising:  
attenuating the hypoxic response of a tissue in said animal by the method of  
Claim 48.

25

50. A method of treating a hypoxia-related condition in a tissue, comprising:  
(a) introducing an expression vector into said tissue; and (b)  
expressing the coding sequence of said expression vector within said tissue,  
wherein said coding sequence is a gene selected from the group consisting of

5 *HIG1, HIG2, annexin V, lipocortin 2, hnRNP A1, Ku autoantigen,*  
*phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7,*  
*fibroblast growth factor-3, EPH receptor ligand, plasminogen activator inhibitor-*  
*1, macrophage migration inhibitory factor, fibronectin receptor, lysyl*  
*hydroxylase-2, endothelin-2, B-cell translocation gene-1, reducing agent and*  
10 *tunicamycin-responsive protein, CDC-like kinase-1, quiescin, growth arrest DNA*  
*damage-inducible protein 45, DEC1, low density lipoprotein receptor related*  
*protein, hamster hairy gene homologue, adipophilin, cyclooxygenase-1, fructose*  
*bisphosphatase, creatine transporter, fatty acid binding protein, lactate*  
*dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1.*

15

51. A method of treating a hypoxia-related condition in a tissue, comprising:  
administering to the tissue a polypeptide expressed by a gene selected from  
the group consisting of *HIG1, HIG2, annexin V, lipocortin 2, hnRNP A1, Ku*  
*autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase,*  
20 *ribosomal L7, fibroblast growth factor-3, EPH receptor ligand, plasminogen*  
*activator inhibitor-1, macrophage migration inhibitory factor, fibronectin*  
*receptor, lysyl hydroxylase-2, endothelin-2, B-cell translocation gene-1, reducing*  
*agent and tunicamycin-responsive protein, CDC-like kinase-1, quiescin, growth*  
*arrest DNA damage-inducible protein 45, DEC1, low density lipoprotein receptor*  
25 *related protein, hamster hairy gene homologue, adipophilin, cyclooxygenase-1,*  
*fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate*  
*dehydrogenase, Bcl-2-interacting killer, Nip3L/Nix, and Pim-1.*

52. A method for identifying stress-inducible genes and fragments of genes, comprising the steps of:

- (a) subjecting one of two populations of cells to stress;
- (b) preparing cDNA libraries from the two populations of cells;
- 5 (c) digesting the cDNA libraries with restriction enzymes and then ligating linker sequences to the ends of said digested cDNA;
- (d) amplifying the cDNA library from the non-stressed cells with tagged primers by means of the polymerase chain reaction and amplifying the other cDNA library from the stressed cells with non-tagged primers by means of the  
10 polymerase chain reaction;
- (e) heating and reannealing the non-tagged, amplified cDNA in the presence of an excess of the tagged, amplified cDNA;
- (f) removing from the mixture those DNA strands which either are themselves tagged or are duplexed with tagged DNA;
- 15 (g) amplifying remaining non-tagged cDNA sequences by means of the polymerase chain reaction;
- (h) repeating, from 0 to 5 times, steps (c) through (g) using as the two cDNA libraries the remaining non-tagged cDNA sequences and the original tagged cDNA library;
- 20 (i) in a separate, second part of the method, performing steps (c) through (h), except that in step (d) the cDNA library from the non-stressed cells are amplified with non-tagged primers and the cDNA library from the stressed cells are amplified with tagged primers;
- (j) in a third part of the method, repeating steps (c) through (g), wherein  
25 in step (c) the two cDNA libraries are the enriched cDNA libraries obtained from the first and second part of this method, and wherein in step (d) the enriched cDNA library from the non-stressed cells is tagged during amplification and the

enriched cDNA library from the stressed cells is amplified with non-tagged primers.

53. The method of Claim 52, wherein said stress is selected from the group  
5 consisting of hypoxia, ionizing radiation, hypothermia, and heat shock.

54. The method of Claim 52, wherein said cDNA libraries of step (b) are prepared with indexed primers.

10 55. The method for identifying stress-repressible genes and fragments of genes, comprising the steps of:

- (a) subjecting one of two populations of cells to stress;
- (b) preparing cDNA libraries from the two populations of cells;
- (c) digesting the cDNA libraries with restriction enzymes and then  
15 ligating linker sequences to the ends of said digested cDNA;
- (d) amplifying the cDNA library from the non-stressed cells with tagged primers by means of the polymerase chain reaction and amplifying the other cDNA library from the stressed cells with non-tagged primers by means of the polymerase chain reaction;
- 20 (e) heating and reannealing the non-tagged, amplified cDNA in the presence of an excess of the tagged, amplified cDNA;
- (f) removing from the mixture those DNA strands which either are themselves tagged or are duplexed with tagged DNA;
- (g) amplifying remaining non-tagged cDNA sequences by means of the  
25 polymerase chain reaction;
- (h) repeating, from 0 to 5 times, steps (c) through (g) using as the two cDNA libraries the remaining non-tagged cDNA sequences and the original tagged cDNA library;

(i) in a separate, second part of the method, performing steps (c) through (h), except that in step (d) the cDNA library from the non-stressed cells are amplified with non-tagged primers and the cDNA library from the stressed cells are amplified with tagged primers;

- 5 (j) in a third part of the method, repeating steps (c) through (g), wherein in step (c) the two cDNA libraries are the enriched cDNA libraries obtained from the first and second part of this method, and wherein in step (d) the enriched cDNA library from the stressed cells is tagged during amplification and the enriched cDNA library from the non-stressed cells is amplified with non-tagged  
10 primers.

56. The method of Claim 55, wherein said stress is selected from the group consisting of hypoxia, ionizing radiation, hypothermia, and heat shock.

- 15 57. The method of Claim 55, wherein said cDNA libraries of step (b) are prepared with indexed primers.

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**FIG 1A**

CGGAAGCCGG TTGGGGTGTG AGAGGTTTTTC TCGCTCTAGG GAGATTCTTC  
AAGCAATCAC TATGTCAACA GACACAGGTG TTTCCCTTCC TTCATATGAG  
GAAGATCAGG GATCAAAACT CATTCGAAAA GCTAAAGAGG CACCATTCTG  
ACCCGTTGGA ATAGCGGGTT TTGCAGCAAT TGTTGCATAT GGATTATATA  
AACTGAAGAG CAGGGGAAAT ACTAAAATGT CCATTCATCT GATCCACATG  
CGTGTGGCAG CCCAAGGCTT TGTTGTAGGA GCAATGACTG TTGGTATGGG  
CTATTCCATG TATCGGGAAT TCTGGGCAAA ACCTAAGCCT TAGAAGAAGA  
GATGCTGTCT TGGTCTTGTT GGAGGAGCTT GCTTTAGTTA GATGCTTTAT  
TATTAAAGTT ACCTATTATT GTTGGAATA AACTAATTTG TATGGGTTTA  
GATGGTAACA TGGCATTTTG AATATTGGCT TCCTTTCTTG CAGGCTTGAT  
TTGCTTGGTG ACCGAATTAC TAGTGACTION TTTACTAACT AGGTCATTCA  
AGGAAGTCAA GTTAACCTAA ACATGTCACC TAAATGCACT TGATGGTGT  
GAAATGTCCA CCTTCTTAAA TTTTAAAGAT GAACTTAGTT CTAAAGAAGA  
TAACAGGCCA ATCCTGAAGG TACTCCCTGT TTGCTGCAGA ATGTCAGATA  
TTTTGGATGT TGCATAAGAG TCCTATTTGC CCCAGTTAAT TCAACTTTTG  
TCTGCCTGTT TTGTGGACTG GCTGGCTCTG TTAGAACTCT GTCCAAAAAG  
TGCATGGAAT ATAACCTGTA AAGCTTCCCA CAATTGACAA TATATATGCA  
TGTGTTTAAA CCAAATCCAG AAAGCTTAAA CAATAGAGCT GCATAATAGT  
ATTTATTAAA GAATCACAAAC TGTAACATG AGAATAACTT AAGGATTCTA  
GTTTAGTTTT TTGTAATTGC AAATTATATT TTTGCTGCTG ATATATTAGA  
ATAATTTTTTA AATGTCATCT TGAAATAGAA ATATGTATTT TAAGCACTCA  
CGCAAAGGTA AATGAACACG TTTTAAATGT GTGTGTTGCT AATTTTTTCC  
ATAAGAATTG TAAACATTGA ACTGAACAAA TTACCTATAA TGGATTTGGT  
TAATGACTTA TGAGCAAGCT GGTTTGGCCA GACAGTATAC CCAAACCTTT  
ATATAATATA CAGAAGGCTA TCACACTTGT GAAATTCTCT TGTCTAATCT  
GAATTTGCAT TCCATGGTGT TAACATGGTA TATGTATTGT TATTAAAGTA  
AGTGACCCAT GTC

**FIG 1B**

MSTD TGVS LP SYEEDQGS KL IRKAKEAPFV PVGIAGFAAI VAYGLYKLKS  
RGNTKMSIHL IHMRVAAQGF VVGAMTVGGMG YSMYREFWAK PKP

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**FIG 2A**

```
ACAAAAC TGG AGTCCACCGC GGTGGCGGCC GCTCTAGAAT AGTGGATCCC
CCGGGCTGCA GGAATTCGGC ACGAGGGCGC TTTTGTCTCC GGTGAGTTTT
GTGGCGGGAA GCTTCTGCGC TGGTGCTTAG TAACCGACTT TCCTCCGGAC
TCCTGCACGA CCTGCTCCTA CAGCCGGCGA TCCACTCCCG GCTGTTCCCC
CGGAGGGTCC AGAGGCCTTT CAGAAGGAGA AGGCAGCTCT GTTTCTCTGC
AGAGGAGTAG GGTCCCTTTC GCCATGAAGC ATGTGTTGAA CCTCTACCTG
TTAGGTGTGG TACTGACCCT ACTCTCCATC TTCGTTAGAG TGATGGAGTC
CCTAGAAGGC TTACTAGAGA GCCCATCGCC TGGGACCTCC TGGACCACCA
GAAGCCAACT AGCCAACACA GAGCCACCA AGGGCCTTCC AGACCATCCA
TCCAGAAGCA TGTGATAAGA CCTCCTTCCA TACTGGCCAT ATTTTGGAAC
ACTGACCTAG ACATGTCCAG ATGGGAGTCC CATTCCTAGC AGACAAGCTG
AGCACCGTTG TAACCAGAGA ACTATTACTA GGCCTTGAAG AACCTGTCTA
ACTGGATGCT CATTGCCTGG GCAAGGCCTG TTTAGGCCGG TTGCGGTGGC
TCATGCCTGT AATCCTAGCA CTTTGGGAGG CTGAGGTGGG TGGATCACCT
GAGGTCAGGA GTTCGAGACC AGCCTCGCCA ACATGGCGAA ACCCATCTC
TACTAAAAAT ACAAAGTTA GCTGGGTGTG GTGGCAGAGG CCTGTAATCC
CAGTTCCTTG GGAGGCTGAG GCGGGAGAAT TGCTTGAACC CGGGGACGGA
GGTTGCAGTG AACCGAGATC GCACTGCTGT ACCCAGCCTG GGCCACAGTG
CAAGACTCCA TCTCAAAAAA AAAAAGAAAA GAAAAAGCCT GTTTAATGCA
CAGGTGTGAG TGGATTGCTT ATGGCTATGA GATAGGTGA TCTCGCCCTT
ACCCCGGGGT CTGGTGTATG CTGTGCTTTC CTCAGCAGTA TGGCTCTGAC
ATCTCTTAGA TGTCCCAACT TCAGCTGTTG GGAGATGGTG ATATTTTCAA
CCCTACTTCC TAAACATCTG TCTGGGGTTC CTTTAGTCTT GAATGTCTTA
TGCTCAATTA TTTGGTGTTG AGCCTCTCTT CCACAAGAGC TCCTCCATGT
TTGGATAGCA GTTGAAGAGG TTGTGTGGGT GGGCTGTTGG GAGTGAGGAT
GGAGTGTTCA GTGCCCATT CTATTTTAC ATTTTAAAGT CGTTCCTCCA
ACATAGTGTG TATTGGTCTG AAGGGGGTGG TGGGATGCCA AAGCCTGCTC
AAGTTATGGA CATTGTGGCC ACCATGTGGC TTAAATGATT TTTTCTAACT
AATAAAGTGG AATATATATT TCAAAAAAAA AAAAAAAAAA CTCGAGGGGG
GCCCCGTACC AATCGC
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**FIG 2B**

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MKHLNLYLL GVLTLISIF VRVMESLEGL LESPSPGTSW TTRSQLANTE
PTKGLPDHPS RSM
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**FIG 3A**

GGCCAGAAAC CGGCAGACTC GGAAGGGACC CCGCGTCTCG GAAGACTCTT  
CAAGAAATCA CAATGTCAAC CAACACAGAC CTTTCTCTCT CTCATACGA  
TGAAGGTCAG GGGTCTAAGT TTATTCGGAA AGCTAAGGAG ACACCGTTTG  
TCCCCATTGG AATGGCGGGC TTTGCAGCGA TTGTTGCCTA TGGGTTGTAC  
AAGCTGAAGA GCAGAGGAAA TACAAAGATG TCCATTCACT TGATCCACAT  
GCGTGTAGCA GCCCAGGGCT TTGTTGTGGG GGCCATGACT CTTGGTATGG  
GCTACTCCAT GTATCAGGAA TTCTGGGCCA ACCCTAAGCC TAAGCCTTAG  
AAGAGCTGGT GGCATGGGAA GTGCTTGCTT TAGTTAGACG TCTCATATTG  
AGGTTACGTG TTTGTATCTA CAATAAATAA CATGTGGGTT TAGA

**FIG 3B**

MSTNTDLSLS SYDEGQGSKE IRKAKETPFV PIGMAGFAAI VAYGLYKLKS  
RGNTKMSIHL IHMRVAAQGF VVGAMTLGMG YSMYQEFWAN PKPKP



**FIG 4A**

```

CGTCAGGCAA AATTACTTCC TCCAGACTGT ACGAGGGATC TGTGGCTCCA
AAGACTCATA AAATAATAAT AATTCTTTAC AGACGATTCA AGAGACACTT
CTTAAACAGT CAGGATGACT GCCTATGATG AGAATGAATC CAAGTTAATG
CGAAAAGTAA AGGAGAATCC ATTTGTCCCA GTGGGGATTG CTGGATTCTT
TGCCATTGTT GGGTACAGAC TGATGAAAAT GAAAAATCGG GGAGACACAA
AAATGTCGGT ACACCTGATC CACATGCGTG TAGCTGCACA AGGCTTTGTG
GTCGGAGCCA TGACTGTTGG AGTCCTGTAT TCAATGTACA GAGATTTTAT
TGTA AAACCC AGAGAAGAAC AGAAATCAAT GCAAAACAAG TGAACACCAC
CTCTTACCCT GGTATTTTAT GTCCCTTAAT ATTACCTCAT ATTAAGGTGT
GTAGAGTGTT TATTTTACT GATGGGTCAA CTTTATATG CACGCATCAC
TCTAGAAGCT CCCCTCTACT GTAAATACCC GTAACCTATT GATCACACTT
GACATCTTCT AAGTATCATA CCAGAGGGTC AAGTTGTCAC TTCTGTATTG
AGAAGGAGTT ATATTGATCT CAGCTGTTTT AACACTGGTT ACACTCCTTG
TGTTTGCGGT TATAAACGTA GCTGGTTTGA TATCTGTGTA GACAGATGAC
ATTACTGAGT GCAGAGTTTT TAGAGCCTCT TATTGTGATG TAGTGTGTGT
GAAATGGCGA GAAGCCTCGA ATTTACGGCA CACGTATCAA TTGTAAACAC
GTATGTCCAT GGCAAAGTCT GGATTTTAAG ACACCCACC AATTGGCAGG
TTGCCCAACA GGTTTCTTCC TCCGGCCGGA ATTTAATGTC TCGTACCAGC
TATATTGTTT TATGTACTAA TTTAGGAACT TTTTGCCAAA TAAAAAATG
CTTGACACTT AGCTCACTTT TTTAATGAGC ATCCCAGTGC ATTTTGGGCA
TCTTGAGGAA GGCTTTGACA ACACTTGACT AACAGACGAA ACCTAAGCTC
CCACATTGTT TAAACAACATA GAACACAAGA GGTTTTTGAC TCACAACAGC
ATCATTTCCAT AAAACAACAT TTTAAATCA TGACATGAAA ATAGGAAATG
GAAAAAAGGA CTATGCATAT TTCTTGACCG AAACATAAAA GATCTCTTGT
AGAATTAAAA TGGATTATTT TAATTTGGTA CGCTTTCCAC AAAATGTCTT
TTTTTTTTTT TGACACAAGG GGGTCCAATA TTTCAATAGA GCAGCTTCAC
AAGCCCTCAC GAGAAATGTA AACCAACTGA CACCTTCACC TGTACAGACT
GTGCAGTAAT CTATAGTATA TCATCATATA GCCTACCTTG TGATAAGCTT
AAATAGATGC CTTGTTAAGT TATACACAAA GTTGAATTTT GAATATTGTG
TGCAAATACA GAAGATGTTA TTGAATGTTT TTTTTCCTC TCGAAATAAA
ATTGACCAGT CTTGTAATTC T

```

**FIG 4B**

```

MTAYDENESK LMRKVKENPF VPGIAGFFA IVGYRLMKMK NRGDTKMSVH
LIHMRVAAQG FVVGAMTVGV LYSMYRDFIV KPREEQKSMQ NK

```

**FIG 5A**

|            |            |            |            |             |
|------------|------------|------------|------------|-------------|
| TCGGACGAGG | GCCTCGCAGA | AGGGCGGGCT | TTGGGAGGTC | CGTTTGTCTT  |
| TGGGGCTTAT | TTCTATCCAG | AGCAGTGCCT | GCGTGGAGCT | TCCACGTTGC  |
| GACTCAGCCG | ACCTTCTTCC | TTACTCCTGC | ACGACCTGGT | GTGACTGTGA  |
| GCAGCCGTCT | CTCAACTTTT | CCTTCTGAGG | ATCTAGCAGC | AGAAAGCAGC  |
| TCTACTTCCC | TGCAAAGGAG | CTGGGCACCG | TCGCCATGAA | GTTTCATGCTG |
| AACCTCTATG | TGCTGGGCAT | CATGTTGACC | CTGCTTTCCA | TCTTTGTTAG  |
| AGTGATGGAG | TCTCTGGGAG | GCTTACTGGA | GAGCCCCTG  | CCCGGGAGCT  |
| CCTGGATCAC | GAGGGGTCAG | CTAGCCAACA | CACAGCCTCC | TAAGGGCCTG  |
| CCAGACCATC | CATCCCGAGG | AGTGCAGTGA | ACCTCCCTCC | CTGCAGGCAT  |
| CACAGCTTCA | GCATGTCCAA | CCACACGTTT | CATTTCTCGG | GAGGCAGCAT  |
| CAAGTGTCTC | CAAAGGACTC | TTACTAGGCC | TGGAAGGGCT | GTTCCCTTAC  |
| CCTGGAAAAG | AGCCTATTTT | CCCTAGAGCT | GTGAGTGGGC | TGTCTGTGGC  |
| TCTGGGATGG | AGGTGTACCA | GTTCCAGCTG | TAGGGAGAAT | GGATTTTGGT  |
| TTGTTTTGTT | TCAGACCTCT | GTCCTAAAGG | ACTCTTTTGG | ACCTAAGTAT  |
| CTTCTGTTGG | TTTACCATTG | AGTCTCTTCC | CTGAGAGTTG | TTTGGATGGC  |
| ATCAAAGGGG | TTGTGGTTTG | ACTGTGAAGA | CAGAGGGTGG | ACTATCCAGT  |
| GTCCAGGTCA | AGTTGTACAT | TTAAGTTCTT | TCTCCAGTGT | AATGCACATG  |
| TGTTTGT    |            |            |            |             |

**FIG 5B**

|             |            |            |            |            |
|-------------|------------|------------|------------|------------|
| MKFMNLNLYVL | GIMLTLLSIF | VRVMESLGGL | LESPLPGSSW | ITRGQLANTQ |
| PPKGLPDHPS  | RGVQ       |            |            |            |

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## FIG 6A

```
hHIG1      1  MSTDTGVSLPSYEEDQGSKLIRKAKEAPFVPVGIAGFAAIVAYGLYKLKS
mHIG1      1  MSTNTDLSLSSYDEGQGSKFIRKAKEIPFVPIGMAGFAAIVAYGLYKLKS
GHL1 (fish). 1  MTAYDENE-SKLMRKVKENPFVPVGIAGFFAIVGYRLMKMKN

hHIG1      51  RGNTKMSIHLIHMRVAAQGFVVGAMTVGMGYSMYREFWAKPKP
mHIG1      51  RGNTKMSIHLIHMRVAAQGFVVGAMTLGMGYSMYQEFWANPKPKP
GHL1 (fish) 42  RGDTKMSVHLIHMRVAAQGFVVGAMTVGVLYSMYRDFIVKPREEQKSMQNK
```

## FIG 6B

```
hHIG2      1  MKHVLNLYLLGVVLTLLSIFVRVMESLEGLESPPGTSWITRSQLANTE
mHIG2      1  MKFMLNLYVLGIMLTLLSIFVRVMESLEGLESPLPGSSWITRQLANTQ

hHIG2      51  PTKGLPDHPSRSM
mHIG2      51  PPKGLPDHPSRGVQ
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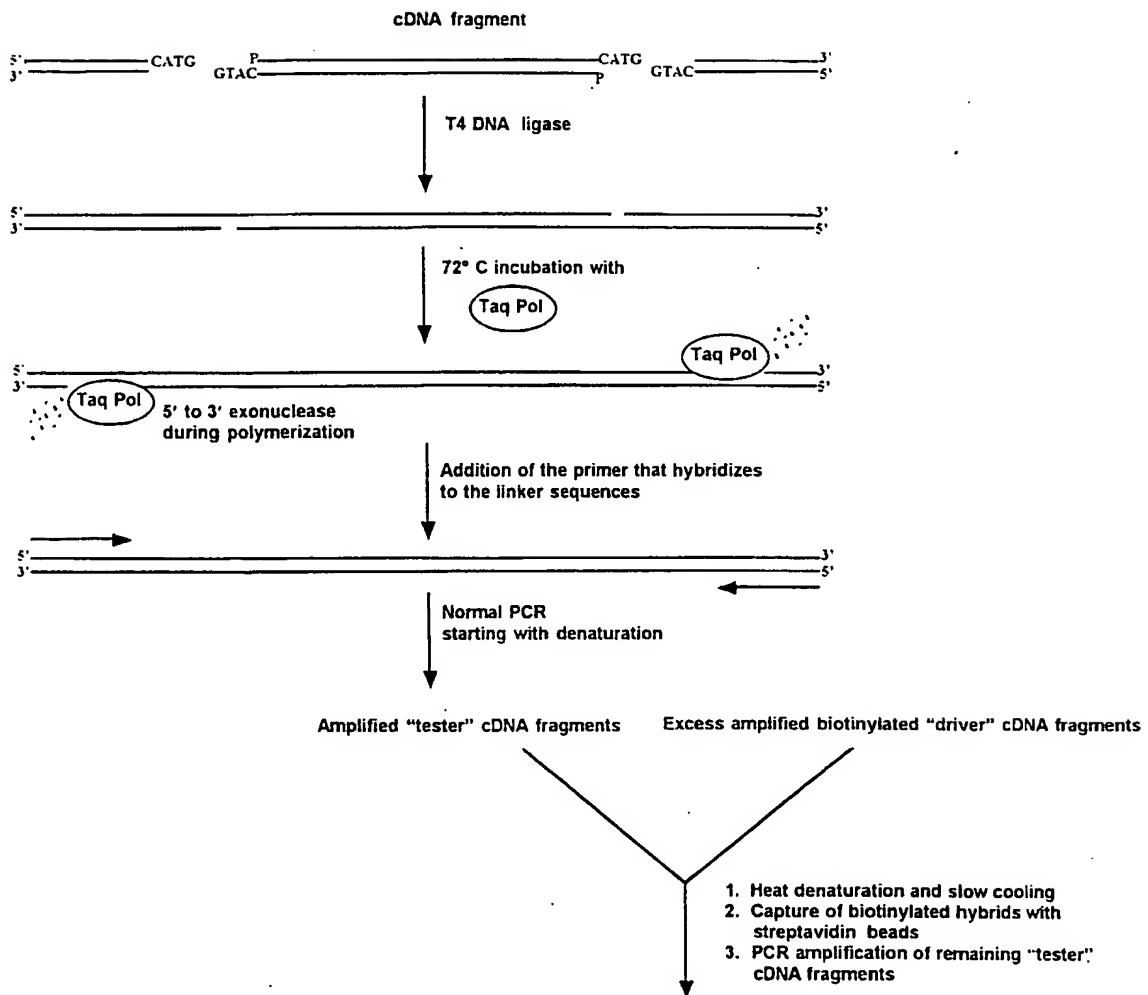
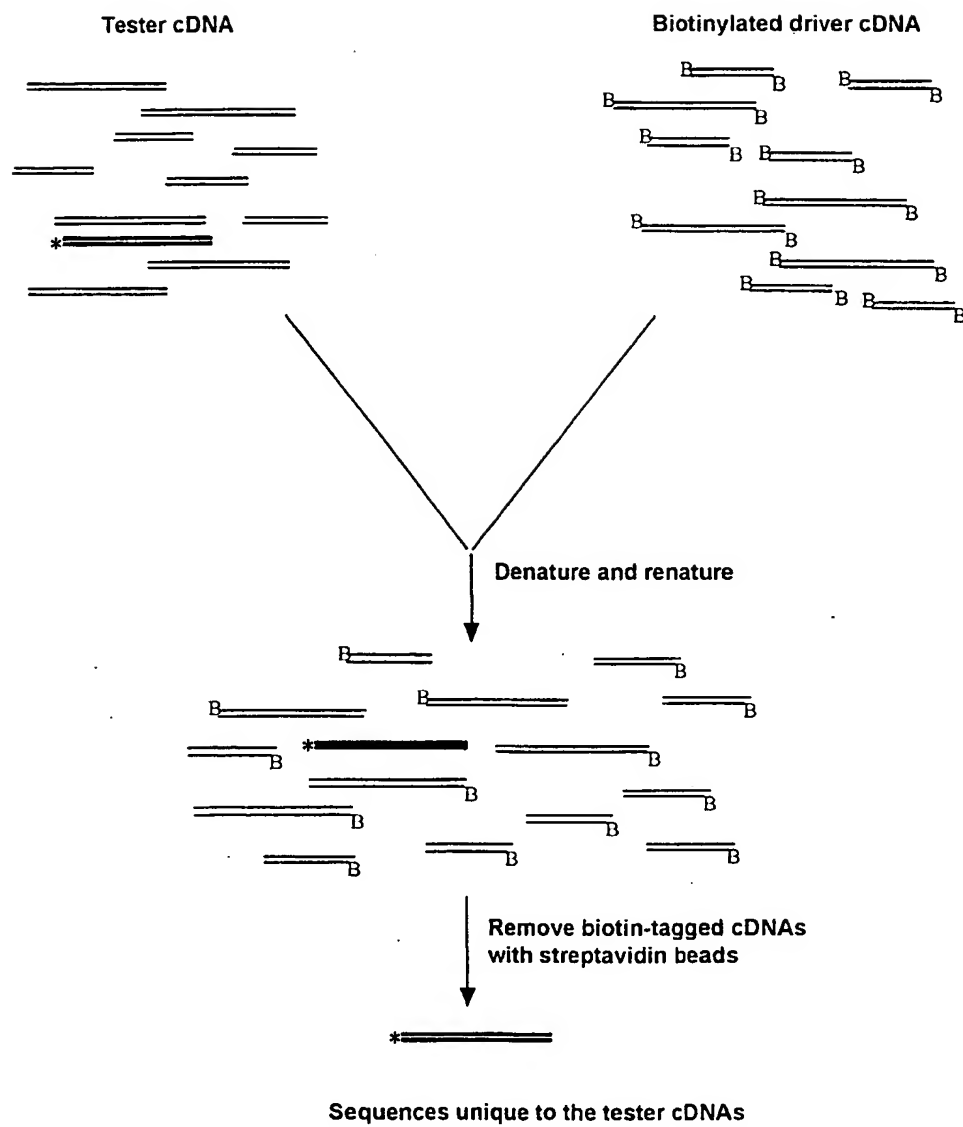


FIG 7

8/8

**FIG 8**

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 Giaccia, Amato J  
 Green, Christopher J  
 Laderoute, Keith R  
 Schindler, Cornelia  
 Koong, Albert C.

<120> Hypoxia-Inducible Human Genes, Proteins, and Uses  
 Thereof

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 Gly Ser Lys Leu Ile Arg Lys Ala Lys Glu Ala Pro Phe Val Pro Val  
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 gga ata gcg ggt ttt gca gca att gtt gca tat gga tta tat aaa ctg 205  
 Gly Ile Ala Gly Phe Ala Ala Ile Val Ala Tyr Gly Leu Tyr Lys Leu  
 35 40 45

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Val Ala Ala Gln Gly Phe Val Val Gly Ala Met Thr Val Gly Met Gly
      65              70              75              80

tat tcc atg tat cgg gaa ttc tgg gca aaa cct aag cct tagaagaaga   350
Tyr Ser Met Tyr Arg Glu Phe Trp Ala Lys Pro Lys Pro
      85              90

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 Gly Ile Ala Gly Phe Ala Ala Ile Val Ala Tyr Gly Leu Tyr Lys Leu  
           35                  40                  45  
 Lys Ser Arg Gly Asn Thr Lys Met Ser Ile His Leu Ile His Met Arg  
           50                  55                  60  
 Val Ala Ala Gln Gly Phe Val Val Gly Ala Met Thr Val Gly Met Gly  
           65                  70                  75                  80  
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| Met Glu Ser Leu Glu Gly Leu Leu Glu Ser Pro Ser Pro Gly Thr Ser    |    |    |      |
| 25   | 30 | 35 |      |
| tgg acc acc aga agc caa cta gcc aac aca gag ccc acc aag ggc ctt    |    |    | 438  |
| Trp Thr Thr Arg Ser Gln Leu Ala Asn Thr Glu Pro Thr Lys Gly Leu    |    |    |      |
| 40   | 45 | 50 | 55   |
| cca gac cat cca tcc aga agc atg tgataagacc tccttcata ctggccatat    |    |    | 492  |
| Pro Asp His Pro Ser Arg Ser Met                                    |    |    |      |
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| caccgttgta accagagaac tattactagg ccttgaagaa cctgtctaac tggatgctca  |    |    | 612  |
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| atggcgaaac cccatctcta ctaaaaatac aaaagttagc tgggtgtggt ggagaggcc   |    |    | 792  |
| tgtaatccca gttccttggg aggctgaggc gggagaattg cttgaacccg gggacggagg  |    |    | 852  |
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&lt;211&gt; 63

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&lt;213&gt; Homo sapiens

&lt;400&gt; 4

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Leu Ser Ile Phe Val Arg Val Met Glu Ser Leu Glu Gly Leu Leu Glu  
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&lt;220&gt;

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 35 40 45

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 35 40 45  
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&lt;211&gt; 92

&lt;212&gt; PRT

<213> *Seriola quinqueradiata*

&lt;400&gt; 8

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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Thr | Ala | Tyr | Asp | Glu | Asn | Glu | Ser | Lys | Leu | Met | Arg | Lys | Val | Lys |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Glu | Asn | Pro | Phe | Val | Pro | Val | Gly | Ile | Ala | Gly | Phe | Phe | Ala | Ile | Val |
|     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gly | Tyr | Arg | Leu | Met | Lys | Met | Lys | Asn | Arg | Gly | Asp | Thr | Lys | Met | Ser |
|     |     | 35  |     |     |     |     | 40  |     |     |     |     | 45  |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Val | His | Leu | Ile | His | Met | Arg | Val | Ala | Ala | Gln | Gly | Phe | Val | Val | Gly |
|     | 50  |     |     |     |     | 55  |     |     |     |     | 60  |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ala | Met | Thr | Val | Gly | Val | Leu | Tyr | Ser | Met | Tyr | Arg | Asp | Phe | Ile | Val |
| 65  |     |     |     |     | 70  |     |     |     |     | 75  |     |     |     | 80  |     |

|     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Lys | Pro | Arg | Glu | Glu | Gln | Lys | Ser | Met | Gln | Asn | Lys |
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&lt;210&gt; 9

&lt;211&gt; 857

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

&lt;220&gt;

&lt;221&gt; CDS

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ttactcctgc acgacctggt gtgactgtga gcagccgtct ctcaactttt ccttctgagg 180

atctagcagc agaaagcagc tctacttccc tgcaaaggag ctgggcaccg tcgcc atg 238

Met

1

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Lys Phe Met Leu Asn Leu Tyr Val Leu Gly Ile Met Leu Thr Leu Leu

5

10

15

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Ser Ile Phe Val Arg Val Met Glu Ser Leu Gly Gly Leu Leu Glu Ser

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25

30

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Pro Leu Pro Gly Ser Ser Trp Ile Thr Arg Gly Gln Leu Ala Asn Thr

35

40

45

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Gln Pro Pro Lys Gly Leu Pro Asp His Pro Ser Arg Gly Val Gln

50

55

60

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&lt;213&gt; Mus musculus

&lt;400&gt; 10

Met Lys Phe Met Leu Asn Leu Tyr Val Leu Gly Ile Met Leu Thr Leu  
 1 5 10 15

Leu Ser Ile Phe Val Arg Val Met Glu Ser Leu Gly Gly Leu Leu Glu  
 20 25 30

Ser Pro Leu Pro Gly Ser Ser Trp Ile Thr Arg Gly Gln Leu Ala Asn  
 35 40 45

Thr Gln Pro Pro Lys Gly Leu Pro Asp His Pro Ser Arg Gly Val Gln  
 50 55 60

&lt;210&gt; 11

&lt;211&gt; 44

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: linker  
 oligonucleotide

&lt;400&gt; 11

ttttaccagc ttattcaatt cggtcctctc gcacaggatg catg

44

&lt;210&gt; 12

&lt;211&gt; 37

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: linker  
 oligonucleotide

&lt;400&gt; 12

catcctgtgc gagaggaccg aattgaataa gctggtgta

37

<210> 13

<211> 45

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: linker  
oligonucleotide

<400> 13

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45

<210> 14

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: linker  
oligonucleotide

<400> 14

catccttccg acttgacgag atactagaat gtctacaa

38

<210> 15

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
oligonucleotide PCR primer

<400> 15

ccagcttatt caattcggtc c

21

<210> 16

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
oligonucleotide PCR primer



<400> 16  
gtagacattc tagtatctcg t 21

<210> 17  
<211> 32  
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<400> 17  
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<210> 18  
<211> 34  
<212> DNA  
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<400> 18  
ggcgcctcgag tctaaacca catgttattt attg 34

<210> 19  
<211> 21  
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<213> Mus musculus

<400> 19  
ccttactcct gcacgacctg g 21

<210> 20  
<211> 32  
<212> DNA  
<213> Mus musculus

<400> 20  
ggcgcctcgag cacatgtgca ttacactgga ga 32

# INTERNATIONAL SEARCH REPORT

Intern. Appl. Application No  
PCT/US 99/06860

|   |  |  |   |  |                      |
|---|--|--|---|--|----------------------|
| <b>A. CLASSIFICATION OF SUBJECT MATTER</b><br>6 C12N15/12 C12N15/70 C07K16/18 A61K31/70 C07K14/435<br>C07K14/47 A61K39/00 A61K48/00 C12Q1/68 G01N33/577<br>C12N15/79  |  |  |   |  |                      |
| According to International Patent Classification (IPC) or to both national classification and IPC   |  |  |   |  |                      |
| <b>B. FIELDS SEARCHED</b><br>Minimum documentation searched (classification system followed by classification symbols)<br>6 C07K  |  |  |   |  |                      |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched   |  |  |   |  |                      |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used)  |  |  |   |  |                      |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>   |  |  |   |  |                      |
| Category * Citation of document, with indication, where appropriate, of the relevant passages   |  |  |   |  | Relevant to claim No |
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| ---<br>-/--   |  |  |   |  |                      |
| <input type="checkbox"/> Further documents are listed in the continuation of box C.   |  |  |   |  |                      |
| <input checked="" type="checkbox"/> Patent family members are listed in annex.  |  |  |   |  |                      |
| * Special categories of cited documents :<br>"A" document defining the general state of the art which is not<br>considered to be of particular relevance<br>"E" earlier document but published on or after the international<br>filing date<br>"L" document which may throw doubts on priority claim(s) or<br>which is cited to establish the publication date of another<br>document or other special reason (as specified)<br>"O" document referring to an oral disclosure, use, exhibition or<br>other means<br>"P" document published prior to the international filing date but<br>after the priority date claimed<br>"T" later document published after the international filing date<br>or priority date and not in conflict with the application but<br>cited to understand the principle or theory underlying the<br>invention<br>"X" document of particular relevance; the claimed invention<br>cannot be considered novel or cannot be considered to<br>involve an inventive step when the document is taken alone<br>"Y" document of particular relevance; the claimed invention<br>cannot be considered to involve an inventive step when the<br>document is combined with one or more other such docu-<br>ments, such combination being obvious to a person skilled<br>in the art.<br>"Z" document member of the same patent family |  |  |   |  |                      |
| Date of the actual completion of the international search<br><br>24 August 1999   |  |  | Date of making of the international search report<br><br>10 NOV. 1999 |  |                      |
| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016   |  |  | Authorized officer<br><br>De Kok, A                                   |  |                      |

# INTERNATIONAL SEARCH REPORT

Intern. Application No  
PCT/US 99/06860

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |   |  |
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| A  | LEVY ET AL: "Regulation of vascular endothelial growth factor by hypoxia and its modulation by the von Hippel-Lindau tumor suppressor gene"<br>KIDNEY INTERNATIONAL,<br>vol. 51, no. 2,<br>1 February 1997 (1997-02-01), pages<br>575-578, XP002083931<br>NEW YORK, NY, US<br>ISSN: 0085-2538<br>abstract<br>---<br>-/--  | 1  |

# INTERNATIONAL SEARCH REPORT

Inten. Application No

PCT/US 99/06860

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 99/06860

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1, 4-14, 41, 42, 45, 48, 50, 51 all partly and 2 completely

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Remark: Although claims 30,36,38,39,40,43,44,46,47,49,50 and 51 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

IT SHOULD BE NOTED, that ONLY those claims for which said compounds/compositions could be identified, i.e claims 50 and 51 have been searched.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1, 4-14,41,42,45,48,50,  
51 all partly and 2 completely

An isolated polynucleotide comprising the sequence set forth in SEQ ID NO:1, the complement thereof, an at least twelve nucleotide-long fragment thereof, or a sequence which hybridizes thereto; an expression vector comprising said polynucleotide; a delivery vehicle comprising said polynucleotide; an isolated cell comprising said polynucleotide; a probe comprising said polynucleotide; an array of polynucleotides comprising said polynucleotide; an isolated polypeptide encoded by said polynucleotide; an array of polypeptides comprising said polypeptide; an antibody immunoreactive with said polypeptide; an array of antibodies comprising said antibody and the use of said polynucleotide, polypeptide, probe, antibody and arrays in diagnosis and treatment of hypoxia-related conditions.

2. Claims: 1, 4-14,41,42,45,48,50,  
51 all partly and 3 completely

An isolated polynucleotide, comprising the sequence set forth in SEQ ID NO:3, the complement thereof, an at least twelve nucleotide-long fragment thereof, or a sequence which hybridizes thereto; an expression vector comprising said polynucleotide; a delivery vehicle comprising said polynucleotide; an isolated cell comprising said polynucleotide; a probe comprising said polynucleotide; an array of polynucleotides comprising said polynucleotide; an isolated polypeptide encoded by said polynucleotide; an array of polypeptides comprising said polypeptide; an antibody immunoreactive with said polypeptide; an array of antibodies comprising said antibody and the use of said polynucleotide, polypeptide, probe, antibody and arrays in diagnosis and treatment of hypoxia-related conditions.

3. Claims: 15-19, 32-35, 37

An array of polynucleotides, comprising at least two hypoxia-inducible genes and its use.

4. Claims: 20-23

An array of polypeptides, comprising the expression products of at least two hypoxia-inducible genes

5. Claims: 24-29, 31

An array of antibodies, comprising at least two different

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

antibodies specifically immunoreactive with the expression products of hypoxia-inducible genes.

6. Claims: 41, 42, 45, all partly

A method of determining the presence of hypoxia comprising assaying the mRNA transcript or the polypeptide expression product of a group of hypoxia-induced genes

7. Claim : 48 partly

A method of attenuating the hypoxic response of a tissue comprising neutralizing a protein selected from a number of hypoxia-induced proteins.

8. Claims: 50, 51, all partly

A method of treating a hypoxia-related condition in a tissue, comprising introducing an expression vector having a coding sequence or a polypeptide in said tissue, wherein said coding sequence or said polypeptide are derived from a group of hypoxia-induced genes.

9. Claims: 52-57

A method for identifying stress-inducible genes and fragments of genes.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/06860

| Patent document<br>cited in search report |   | Publication<br>date |    | Patent family<br>member(s) |  | Publication<br>date |
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Information on patent family members

International Application No

PCT/US 99/06860

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
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